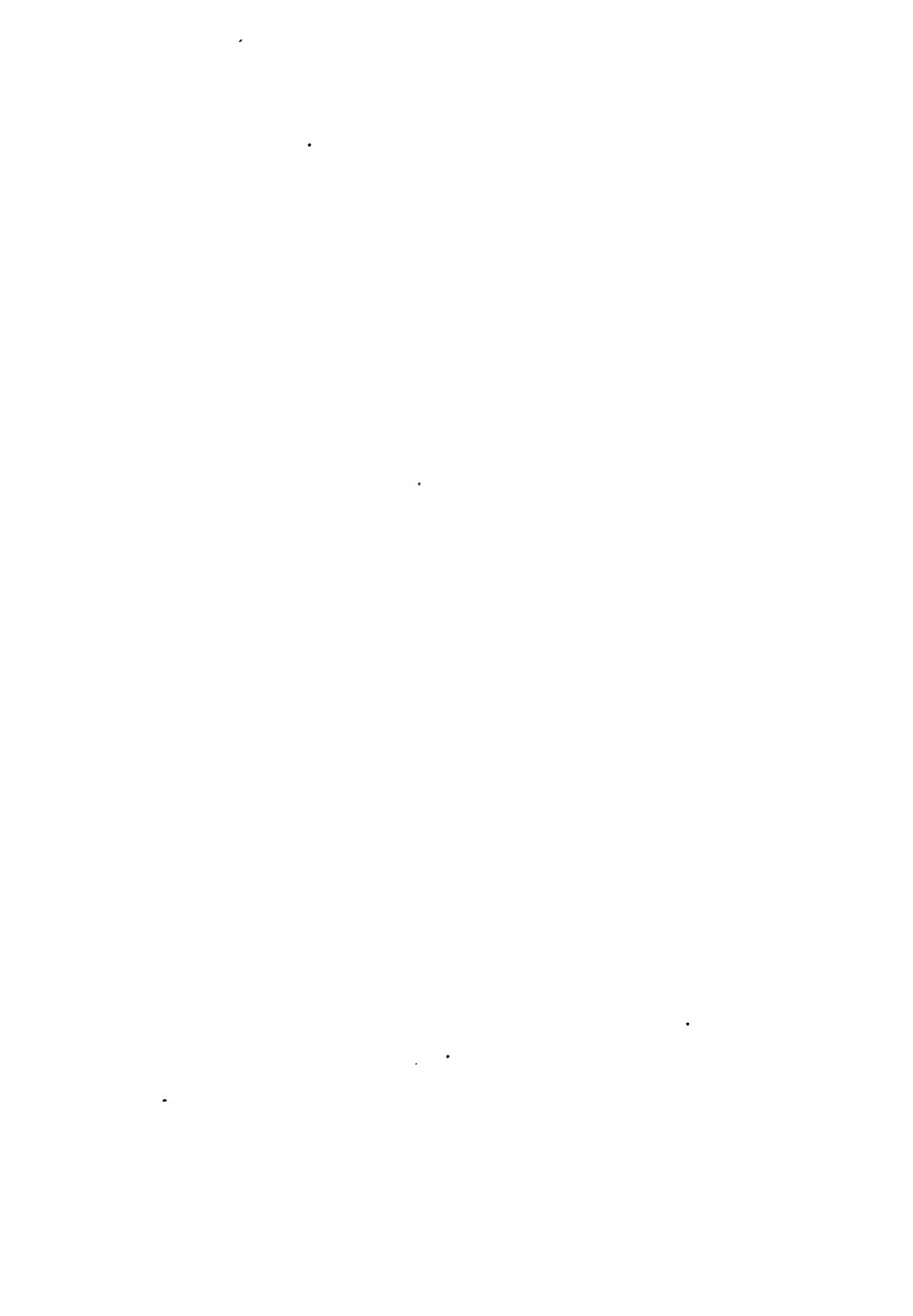


ACTA PATHOLOGICA ET MICROBIOLOGICA
SCANDINAVICA
VOL. XXV



ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

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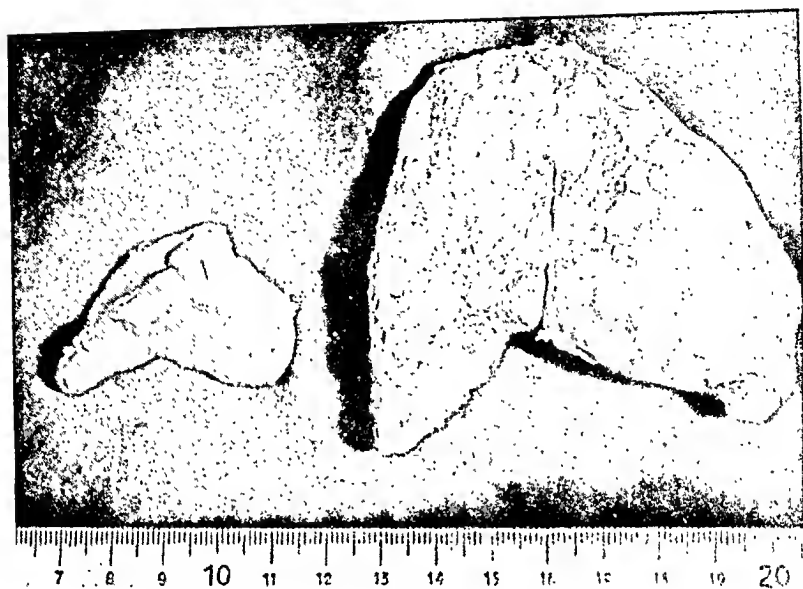


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Autopsy preparation of enlarged adrenal (right)
compared with normal (left)



Monday July 7th, 1947.

Meeting of Directors of the Scandinavian Pathological Society, for discussion of matters relating to the general assembly during this meeting and the following.

The congress was inaugurated by the President, Professor Robin Fåhræus (Uppsala), who delivered an address in memory of members of the Scandinavian Pathological Society deceased since the last meeting, Lector *Vilhelm Jensen* (Denmark), Dr. *Arnoldus Schytte* (Norway), Veterinary Inspector *C. W. Andersen* (Denmark), Professor *Harry Castrén* (Finland), Professor *Carl Nyberg* (Finland), Chief Pathologist *Ole Berner* (Norway), Professor *Axel Wallgren* (Finland), Professor *John Forssman* (Sweden).

The following *vice-presidents* were elected:

Section of Pathology: Professor *H. Bergstrand* (Stockholm), Professor *O. Brandt* (Oslo), Professor *A. Hjärre* (Stockholm), Dr. *T. Kemp* (Copenhagen), Professor *A. Saxén* (Helsingfors).

Section of Bacteriology: Chief Physician *L. Borgen* (Oslo), Head of Department *M. Kristensen* (Copenhagen), Professor *A. Lindau* (Lund), Professor *G. Olin* (Stockholm), Arkiater *O. Renkonen* (Helsingfors).

The following secretaries were elected:

Chief Physician *F. Wahlgren*, Secretary General (Stockholm).

Section of Pathology: Dr. *N. O. Christensen* (Copenhagen), Laborator *A. Isaksson* (Stockholm), Docent *N. Ringertz* (Stockholm), Docent *H. Teir* (Helsingfors), Pathologist *O. Torgersen* (Oslo).

Section of Bacteriology: Head of Department *P. Holm* (Copenhagen), Docent *B. Malmgren* (Stockholm), Dr. *Ö. Ouchterlony* (Stockholm), Docent *J. Pikkarainen* (Helsingfors), Chief Physician *T. Vogelsang* (Bergen).

It was resolved thankfully to accept the invitation from the Finnish Pathological Society — presented by Chief Pathologist *I. Wallgren* — to hold the next Scandinavian Pathological Congress at Helsingfors in 1950.

PRESENT AT THE MEETING:

DENMARK:

Andersen, H., Copenhagen.
 Bjerneboe, M., Copenhagen.
 Christensen, Erna, Copenhagen.
 Christensen, H., Copenhagen.
 Christensen, N. O., Copenhagen.
 Clemmesen, J., Copenhagen.
 Dragsted, P. J., Frederiksberg.
 Engelbreth-Holm, J., Copenhagen.
 Eriksen, K. R., Copenhagen.
 Eskelund, V., Copenhagen.
 Frederiksen, O., Copenhagen.
 Folger, A. F., Charlottenlund.
 Hansen, J. L., Copenhagen.
 Heerup, L., Copenhagen.
 Hogrefte, G., Copenhagen.
 Jensen, K. A., Copenhagen.
 Kemp, T., Copenhagen.
 Krag, P., Copenhagen.
 Kristensen, M., Copenhagen.
 Lester, Vera, Copenhagen.
 Langhorn, A., Copenhagen.
 Maaloe, O., Copenhagen.
 Magnus, Herdis von, Copenhagen.
 Magnus, P. von, Copenhagen.
 Ohlsen, A. Soeborg, Gentofte.
 Rask-Nielsen, Ragna, Copenhagen.
 Schambye, P., Copenhagen.
 Scheibel, Inga, Copenhagen.
 Seeman, H., Copenhagen.
 Sjolte, I. P., Copenhagen.
 Teilum, G., Copenhagen.
 Tolderlund, K., Copenhagen.
 Tulinius, S., Copenhagen.

FINLAND:

Ahvenainen, E. K., Helsingfors.
 Järvi, O., Åbo.
 Pikkarainen, J., Helsingfors.
 Renkonen, O., Hietanen.
 Saxén, A., Helsingfors.
 Saxén, E., Helsingfors.
 Setälä, K., Munksnäs.
 Sourander, P., Åbo.
 Stenius, P. I., Helsingfors.
 Teir, H., Helsingfors.
 Uotila, U., Helsingfors.
 Wallgren, I., Helsingfors.
 Wangel, G., Helsingfors.

NORWAY:

Borgen, L. O., Oslo.
 Brandt, A., Oslo.
 Christie, A., Oslo.

Foss, B., V. Aker.
 Hval, E., Oslo.
 Kreyberg, L., Oslo.
 Refvem, O., Oslo.
 Svendsen, M., Oslo.
 Torgersen, O., Oslo.
 Waaler, G., Oslo.
 Vogelsang, T., Bergen.

SWEDEN:

Ahlström, C. G., Lund.
 Berg, N. O., Lund.
 Bergqvist, S., Stockholm.
 Bergstrand, A., Stockholm.
 Bergstrand, H., Stockholm.
 Davide, H., Stockholm.
 Fagréus, Astrid, Stockholm.
 Fähræus, R., Uppsala.
 Gard, S., Stockholm.
 Gellerstedt, N., Uppsala.
 Gullbring, B., Stockholm.
 Hallberg, V., Uppsala.
 Hansen, H.-J., Stockholm.
 Hansson, Hild., Stockholm.
 Hedén, C.-G., Stockholm.
 Hedlund, P. G., Stockholm.
 Henschen, F., Stockholm.
 Hjärre, A., Djursholm.
 Hultquist, G., Stockholm.
 Isaksson, A., Stockholm.
 Lindau, A., Lund.
 Lindholm, O. E., Uppsala.
 Magnusson, H., Stockholm.
 Malingren, B., Stockholm.
 Mellgren, J., Lund.
 Olin, G., Stockholm.
 Ouchterlony, Ö., Stockholm.
 Packalén, T., Stockholm.
 Ranström, S., Uppsala.
 Reuterwall, O., Stockholm.
 Ringertz, N., Stockholm.
 Rubarth, C. S., Stockholm.
 Sievers, O., Göteborg.
 Thorell, B., Stockholm.
 Thulin, K. E., Lund.
 Wahlgren, Fr., Stockholm.
 Wallenius, G., Uppsala.
 Vejlens, G., Göteborg.
 Westin, G., Stockholm.
 Wilman, G., Stockholm.
 Wilton, A., Stockholm.
 Winblad, S., Malmö.
 Wählin, O. S., Uppsala.
 Zetterberg, B., Uppsala.
 Zettergren, L., Uppsala.

Papers read before the congress July 7-9th, 1947

1. Section of Pathology

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<i>Clemmesen, Johannes</i> : The Danish cancer registry. Problems and results	26
<i>Ahlström, Carl Gustaf</i> : Fluorescence-optical studies on the absorption of various cancerogenic hydrocarbons in the skin. Manuscript not received. Discussion, see under Berg.	
<i>Berg, N. O.</i> : Comparison of epidermal hyperplasia in the skin of mice after application of carcinogenic and noncarcinogenic irritants	34
<i>Teir, Harald</i> : On colchicine tests for the purpose of ascertaining cell division and regenerative conditions in the liver of the rat	45
<i>Christensen, Erna</i> : On tumors of peripheral nerves with a special view to neurogenous sarcomas. Published in <i>acta psych. et neurol. Suppl.</i> 46, 72, 1947.	

After a brief historical survey, a review is given of the case histories of 6 patients with peripheral nerve tumors, 2 of them with neurinoma, and 4 with neurogenous sarcoma. Two of the latter patients had recurrence of the tumor with malignant degeneration. The best therapeutic result is obtained with simultaneous X-ray treatment and extirpation.

<i>Thorell, B.</i> : The relation of the synthesis of hemoglobin to the cellular growth during normal and certain pathological conditions	54
<i>Saxén, Erkki</i> : Tumours of tactile end-organs	66
<i>Hogreffe, Georg</i> : Experimental-genetic studies on leucemia in mice	80
<i>Wallgren, I.</i> : On the cytology of the erythropoiesis. To be published in the proceedings of the VI international congress for experimental cytology, Stockholm 1947.	
<i>Ranström, Stig</i> : Erythroblastosis foetalis.	

Discussion of the clinical symptoms on the background of the pathologic-anatomical changes in fetal erythroblastosis, together with considerations of the genesis of the disease.

Discussion.

WILTON, A.: As a pathologist to the pediatric clinics of the Karolinska Institutet I have had occasion to examine several cases of fetal erythroblastosis, and I have also made some animal experiments on flooding the peripheral blood with erythroblasts. As I have performed autopsy on some severe cases of erythroblastosis that terminated fatally a few days after delivery, I am unable to subscribe to the view advanced by Dr. Ranström, that the Rh factor enters the mother's blood only after labor has commenced. For it is difficult to see how the antibody formation in the mother may be brought about in such a short time; and the same applies to the hemolysis in the child, with the resulting reactive changes in the spleen, liver, adrenals, skin and other organs.

However, the concept of erythroblastosis is somewhat diffuse, and Dr. Ranström gave no definition of his conception of erythroblastosis. Originally, indeed, this term referred to certain morbid conditions in the newborn, with severe icterus, congenital hydrops, etc. associated with erythroblastemia. The term further implied that the provocative factor should be unknown. I have seen such morbid condition induced, for instance, by tuberculosis or syphilis in the mother. These cases were classified by the pediatricians after the mother's illness, and they were not designated as erythroblastosis, as the disease-producing factor was known. I do not know whether this definition still is valid. If so, I think, it would hardly be correct to apply the term of erythroblastosis to cases of severe anemia induced by Rh antibodies, as in such cases the anemia-provoking factor is known.

As to the genetic considerations outlined by Dr. Ranström. I should like to ask whether the changes in the blood and organs observed in erythroblastosis and similar morbid conditions in the newborn might not be explained as resulting from lack of hemoglobin in the blood stream. Really the presence of erythroblasts in the blood stream means only that the hemoglobin reserve of the organism in the form of non-nucleated reticulocytes in the bone-marrow has been used up, more or less, and that the hemoglobin requirement in the blood stream therefore has to be covered by means of erythroblasts.

My own studies of the development of the physiological blood cell have shown that the above-mentioned hemoglobin reserve in the bone marrow increases with the progressing ontogenetic development. This may be the reason why nucleated red blood cells are found more often in anemia in the fetus and newborn than in adults. In my opinion, erythroblastemia is not a phenomenon specific of a certain form of anemia or other morbid condition but it signifies a lack of hemoglobin in the blood stream at an early stage of the ontogenetic development.

As to icterus gravis and hydrops, indeed, these changes are explained by the hemolysis — whether this be due to the change from intrauterine to extrauterine life, the Rh factor or other factors leading to increased destruction of red blood cells.

HULTQUIST, G.: I should like to ask Dr. Ranström how we are to explain that the same pathologic-anatomical features of erythroblastosis are seen in children delivered by cesarean section as in children born by spontaneous delivery. Indeed, cesarean section ought to eliminate the risk of the placental injuries, which Wiener assumes to arise from labor.

In a material comprising 15 cases of erythroblastosis foetalis that I have collected the occurrence of disturbances in the pathologic-anatomical picture of the endocrine glands has not been so common as perhaps suggested by Dr. Ranström's paper. Thus, for instance, only about one-fifth of the cases showed a distinct hyperplasia of the pancreatic islands.

HEERUP, L.: On examinations of placental tissue from abortions, in the cases of spontaneous abortion no vessels are seen in the chorionic villi. In many cases, however, all the erythrocytes in the vascularized chorionic villi are seen to be nucleated, whereas in other cases no nucleated erythrocytes are seen at all.

It is conceivable that several spontaneous abortions may be due to an early erythroblastosis, and does the literature give any information about this question?

- Wihman, G.: Cytological findings in exudates and transudates ... 87
 Bergstrand, Hilding: Hypertensive vascular changes in the eye ... 98
 Foss, B.: Experimental anaphylactic iridocyclitis.

The reaction of the eye in intrabulbar injection of foreign serum under different conditions. The reaction of the sensitised eye to injections of homologous antigen. The capacity of the eye for sensitization of the organism as compared to that of the skin. Demonstration of total preparations and lantern slides.

Discussion.

AHLSTRÖM, C. G.: It was of particular interest that Dr. Foss was able to produce an exacerbation of allergic iridocyclitis by intravenous administration of the allergen after a first reinjection had been given in the eye. Localization of the allergic reaction may be induced in several ways in the skin: heating, chilling, toxic substances.

By means of very small doses of staphylo toxin I have tried to localise a serum allergic reaction to the kidneys by intravenous injection of the serum.

I should like to ask Dr. Foss whether he has tried through unspecific irritants to localise the allergic reaction to the eye by intravenous injection of serum.

FOSS, B.: In an experiment that lasted over 18 months, two animals were given *human serum* in the left eye, and had their primary anaphylactic iridocyclitis. 14 months later both animals were given 5 cc. horse serum without showing any reaction from the left eye. Two months later the animals were given *horse serum* in their right eye, and after a few hours they had their reactive iridocyclitis. Two months later the animals were given *human serum* intravenously. Now I expected a reaction from the left eye, but there appeared a secondary anaphylactic reaction from their *right* eye. What had now happened?

It is conceivable that a doubling of unspecific antigens has taken place in the right eye. But I have also imagined that antibody from human serum has been displaced from the cells and entered the circulation. Through the congestion of the right eye, I imagined that this circulating antibody might be deposited by being led out through the capillary wall. In other words, the antibody has been moved from one eye to the other.

Henschen, Folke: Intravascular tubercle formation.

Reactions of the vascular endothelium in tuberculosis and syphilis are known but slightly. Syphilitic endocapillaritis was demonstrated by me in 1923, endovascular tubercle formation in 1929. Mandelstamm takes my interpretation of these structures in tuberculosis to be erroneous. Revision of older specimens and studies on new ones have shown my first interpretation to be correct. — The relationship of these phenomena to other vascular reactions is pointed out.

- Järvi, Osmo:* Methods for staining of membranous structures and hyalin in the kidneys. No abstract received.
- Saxén, Arno:* On granuloma produced by talcum.

Experimental and clinical studies on injuries to tissues brought about by talcum and talcum-containing powders employed in the treatment of wounds.

Discussion.

CLEMMESSEN, JOHANNES: I should like to ask whether we might reasonably assume a general allergic reaction to talcum. Together with Dr. Helge Faber I have had occasion to observe the case of a patient who presented nodular granulomas, which were reddish in colour and tender to touch. These nodules were located on one knee, which had been »bad« in childhood and also in a scar after the laparotomy which was performed 10 years prior to the present admission.

Microscopic examination showed »lipoid granulomas« in both places. In the abdominal affections, polarization microscopy revealed the presence of crystals resembling talcum. I wonder whether a sensitization with following hypersensitiveness may constitute the basis for the changes here observed.

- Refvem, Olav:* I. Chronic granulomas in the alimentary tract caused by minute mineral particles. II. »Boeck's disease« and occurrence of minute mineral particles 107
- Rubarth, S.:* The pathologicohistologic liver picture in contagious hepatitis in dogs 122
- Hansen, Jens.:* Autopsy statistics on peptic or duodenal ulcer — new Danish experiences.

Data on the occurrence of the disease in a fairly large autopsy material from Copenhagen in 1937—43 — a continuation of the author's previous investigations presented in a book: »Ændringer i Ulcus sygdommens Fremtræden (1907—1936), belyst ved en sektionsstatistisk Methode«. Munksgaard, Copenhagen.

Discussion.

WILTON, Å.: To me Dr. Hansen's grouping of the peptic affections into essential and accidental lesions seems expedient and serving the purpose, as it offers a greater possibility of estimating correctly, for instance, the mortality percentage.

I should like to ask Dr. Hansen, however, whether in his autopsy-statistical calculations he has considered the biological difference between ulcer and scar. This difference may indeed be said to consist in this: the ulcer may disappear by being transformed into a scar, whereas practically all scars remain persistently. The number of scars will then increase with increasing age. Quite recently I have studied Falconér's statistical account of peptic lesions in the autopsy material from St. Erik's Hospital in Stockholm. From this the scar frequency in women appeared continuously to increase with the age. In men, on the other hand, the scar frequency showed a significant fall for the age-class of 60—70 years. This may indeed be due to the circumstance that scars in men previously were less common than now, which would lower the frequency of scars in old men. Something similar would turn out if the lethality previously were greater than now, or if the ulcers appeared in combination with diseases having a higher lethality for the age-class of 50—60 years than for that of 60—70. I wonder whether these factors of selection may have been of any

significance to the mortality calculations carried out by Dr. Hansen.

Then we may consider the tendency to healing. After previous German authors, indeed, attempts have been made to estimate this tendency after the principle: the more scars in proportion to the ulcers encountered in the autopsy material, the greater is the tendency to healing. Numerically this principle is converted so that the so-called healing percentage is calculated as the quotient between scars and ulcers + scars. But the fact has been overlooked that the number of scars increases with increasing age — which does not apply to the ulcers. The error in such a principle of calculation may be illustrated most plainly by means of autopsy material from a children's hospital. In children an ulcer may be found now and then, but never scars. After the above principle of calculation the healing percentage for the children will be 0, signifying that ulcers would never heal in children. Furthermore, all the scars met with in patients belonging, for instance, to the age-class of 70—80 years might come from the healing of ulcers at a much earlier age. Assuming, for instance, that a chronic ulcer never heals after the age of 70, the healing percentage after this age would then be 0. Nevertheless, using the above principle of calculation for the healing tendency we will get a high value.

In the aforementioned statistical account Falconér has also tried to calculate the healing tendency of acute ulcers. The healing percentage was calculated as the quotient between acute ulcers and acute+chronic ulcers. Naturally the values obtained through such calculations must be misleading, as no allowance is made for the fact that the healing time for an acute ulcer is considerably shorter than that for a chronic ulcer, and that chronic ulcers more often terminate fatally than do acute ulcers. Supposing, for instance, that the healing time is one month for acute ulcers, two years for chronic ones. In an autopsy material, then, the chronic ulcers will be 24 times as frequent as the acute. Moreover, when the lethality is higher for chronic ulcers than for the acute ones, the resulting overrepresentation of chronic ulcers will have to be taken into consideration too.

From these remarks, I think, it will be evident that the autopsy-statistical calculations which now are being employed to determine the healing percentage of peptic lesions are not correct.

Also other factors of selection have to be taken into account — *e. g.*, shifts in the age and sex distribution of the autopsy material. The statistical data presented by Dr. Hansen go as far back as to 1907, when the average lifetime may safely be assumed to have been shorter than now. If the mean lifetime of the autopsy material has increased, and if the scars for the above mentioned reasons increase in frequency with increasing age, the frequency of the scars in his material is bound to increase. If the value varies with the age of the patients, in general, I think, it will be safe to say that all such values without correction for the age will be of only slight value or none at all. As I touched upon also yesterday, this applies not only to scars after peptic ulcer but also, for instance, to cancer of the stomach.

Nor is Dr. Hansen's autopsy material homogeneous as far as the sex distribution is concerned, as there has been a shift in this way that the relative frequency of women has become greater. As the figures for the frequency of men and women differ in the various periods, the comparisons carried out for both sexes together cannot be correct.

Further, another source of error is found in the interest taken by the various pathologists in the registration of peptic lesions. Dr. Hansen has tried to eliminate this source of error by cal-

culating also the frequency of registered duodenal diverticula round the papilla of Vater, in which no increase in the frequency could be demonstrated. Such methods for control of the sources of error mentioned deserve all sorts of recognition. From the figures obtained from St. Erik's Hospital, however, it is evident how great a rôle the search of the pathologists for peptic lesions may play in the figures for the frequency. The great increase in the number of peptic lesions registered in this autopsy material during the last decade is undoubtedly attributable in no slight degree to the interest taken by the pathologists in such registration.

The objections I just have raised apply more to the biological sources of error than to the statistical. It will be appropriate, I think, to illustrate this further. Here, for instance, in the table presented by Dr. Hansen we noticed a great rise in the frequency of essential pyloric and duodenal ulcers in men. Indeed, the figures are surprising. If I understood Dr. Hansen correctly, however, in his calculation of these figures he has taken for granted that the healing time and the lethality have been the same in the period under comparison. But, many investigators of peptic lesions — including, among others Swedish authors — have tried to show that this disease has been aggravated so that the healing now takes longer, and there has been an increase in the number of fatal cases. Let us assume that this is correct. Let us further assume, for instance, that a duodenal ulcer which previously took a year to heal, now takes two years, and that the number of fatal cases has been increased by 50 %. Such a change in the ulcer disease would naturally lead to an increase in the frequency for an autopsy material without necessarily meaning that the number of new cases had increased. With these remarks I have wished merely to illustrate some of the difficulties and sources of error associated with conclusions that are based on autopsy statistics. The point is to pay close attention to the factors of selection involved, and they are not few in number. Also Dr. Hansen has done this on some important points, and I wish to state that his autopsy-statistical works on peptic ulcer are among the best ones on the subject that I have read.

Vejlens, Geert: Eosinophilia in peptic ulcer.

Large amounts of eosinophil blood cells accumulate round the peptic ulcer. The eosinophilia here as well as in other morbid conditions is interpreted as a defensive reaction of the body against substances irritating the parasympathetic system, *e. g.*, acetylcholine.

Discussion.

ASK-UPMARK: I have been particularly interested in that part of Dr. Vejzens' paper where clinically certain features of peptic ulcer appear possibly to be accompanied by the presence, for instance, of an allergic factor in the genesis of the ulcer. For one thing some ulcer patients have asthma and ulcer alternatingly. In the next place I wish to point out the remarkable fact that peptic ulcer represents a localised, circumscribed injury. If, as seems reasonable, we assume the peptic factor to be of decisive importance in the genesis of the ulcer we should expect an alteration of a greater or lesser part of the entire »Magenstrasse« region.

Just a small remark about the Dr. Vejzens' presentation of the problem: histamine is no stimulant of the parasympathetic system. The gastric secretion after injection of histamine represents nearly an increase in the output of hydrochloric acid, not of pepsin. Real parasympathetic stomach juice is obtained through injection of insulin — through the resulting hypogly-

emia, and is characterized not only by the increase in acid but also by increased output of pepsin — and probably the same applies to the findings on injection of acetylcholine and of histamine.

WALLGREN, L.: I too have found a very frequent occurrence of eosinophil granulocytes in several cases of peptic ulcer, but numerous eosinophil cells are also found in carcinoma of the stomach, and this disease, I think, may hardly be conceived as being of allergic origin. A feature common to both of these diseases, however, is the appearance of ulcerative processes, implying a possibility of absorption of substances foreign to the organism. One wonders what function the eosinophil cells may have in such conditions. Do they produce any secretion?

In the living eosinophil cells we find no droplets that might be looked upon as secretory droplets. Still, this does not go against the possibility of a secretion. In the heparinocyte we meet with a parallel phenomenon. This cell has a cytoplasmic structure that greatly reminds of a corresponding structure in the eosinophil granulocyte. Judging from the stainability, heparin is concentrated to the less refractive drop system in the cytoplasm, and it can be given off to the surroundings. In a living, unstained, cell the corresponding morphological changes thus should not be noticeable. From a secreting free cell, indeed, the secretion may be given off to the surroundings already before it has been gathered into droplets.

AHLSTRÖM, C. G.: According to my experiences the occurrence of eosinophil leukocytes at the bottom of the ulcer is variable, being sometimes plentiful, sometimes scanty, probably depending on various conditions for healing, local tissue absorption etc. The finding of eosinophil leukocytes in peptic ulcer, however, is difficult to estimate as an allergic phenomenon, as these leukocytes normally occur in great numbers in the mucous membrane of the digestive tract — presumably as an expression of absorptive processes. If, on the other hand, we wished to take the finding of eosinophil leukocytes at the bottom of the ulcer as evidence of an allergic reaction, I wish to point out that also the fibrinoid degeneration often presented by the bottom of the ulcer may be interpreted in the same direction. It is doubtful, however, whether it really be justified on the basis of these findings to classify peptic ulcer as an allergic disease.

<i>Torgersen, Olan:</i> Islets of Langerhans in fibrocytic disease of the pancreas, a hitherto undescribed abnormality?	124
<i>Hultquist, Gösta:</i> An investigation on pregnancy in diabetic animals	131
<i>Bergstrand, Anders:</i> A case of diabetogenic dwarfism	141
<i>Svensen, Magne:</i> On the ordinary lateral inguinal hernia, its heredity and connection with the sex ratio at birth.	

On the basis of a collected material comprising 1861 operated cases of lateral inguinal hernia it is shown that inguinal hernia is due to a dominant hereditary factor which in double dose is lethal in female fetuses. Owing to this hyperbathality the normal sex ratio at birth is 105 boys per 100 girls.

<i>Mellgren, Jan:</i> Experimental studies on the morphology of the pituitary in hyperfunction and hypofunction of the adrenal cortex in rats. To be published in <i>Acta path. et microbiol. scand.</i>	
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Ahvenainen, E. K.: On aspiration of amniotic fluid.

Microscopic examinations of autopsy material elucidating aspiration of amniotic fluid in children born at term and in premature children. Development of »vernix membranes« and their significance.

Discussion.

GELLERSTEDT, N.: No abstract received.

AHVENAINEN, E. K.: To Dr. Gellerstedt I wish to state that transitional forms are found between elements of the amniotic fluid and coherent membranes. It may be that also other substances are present in the membranes, but they are formed chiefly of amniotic fluid. Also other membranes are encountered that have not originated from amniotic fluid.

Fåhræus, R.: Dr. S. E. Björkman's investigations on the circulation in the spleen. No abstract received.

Wahlgren, F. and S. Löfgren: Histopathological studies on erythema nodosum, to be published in *acta dermato-venereologica*.

Discussion.

WILTON, A.: Regarding the remark of Professor Bergstrand that the most characteristic feature of allergic tissue reactions is the so-called fibrinoid reaction, I wish to point out that this change must not be looked upon as specific. Indeed, such changes are encountered in morbid conditions which certainly are not allergic — *e. g.*, scurvy.

A characteristic of the fibrinoid reaction — as we have seen in the beautiful pictures presented by Professor Bergstrand — is the division of the collagenic fibers of the connective tissue into delicate fibrils which fail to stain like ordinary connective tissue fibrils, while they turn black on treatment with silver salts. On study of the connective tissue cells in such foci of degeneration, they often are found to show changes typical of immature cells, *e. g.*, basophilia of the cytoplasm, vesicular nuclei with large nucleoli, etc. On this account I have interpreted the fibrinoid degeneration as a process of dedifferentiation — and I shall briefly outline what I mean with this expression.

Immature connective tissue cells are able only to produce delicate, so-called precollagenic fibers that contain no collagen. Therefore they fail to stain after the ordinary methods for the staining of collagen, whereas they are stained black with silver-salts. When the connective tissue cells attain a higher degree of maturity they also become able to produce collagen. The collagen formation results in a homogenization of the fine precollagenic fibrils, giving rise to more coarse fibrils of stainable collagen. If now a noxious factor — *e. g.*, vitamin C deficiency — gives rise to such a disturbance of the cell metabolism that the connective tissue cells return to a functionally immature stage — *i. e.*, undergo dedifferentiation — the cells lose their capacity for maintaining the collagenic deposits already made. The collagen dissolves and disappears, and the delicate precollagenic fibrils may now be observed once more. Provided that the process of dedifferentiation has not been going on too long, it is reversible. This can be demonstrated most simply by giving vitamin C to the scorbutic animal, in which the dedifferentiated cells then are seen to resume their previous degree of functional

maturity. Collagen is deposited anew, and the precollagenic fibrils disappear. Naturally such a process of dedifferentiation may be induced by several noxious factors, and hence the so-called fibrinoid degeneration must not be taken as specific of allergic tissue reactions.

BERGSTRAND, H.: No abstract received.

WILTON, A.: I quite subscribe to the view advocated by Professor Bergstrand, that the fibrinoid reaction in peptic ulcer cannot be looked upon as signifying an allergic tissue reaction. Earlier to-day, however, peptic ulcer as an allergic disease has been under discussion just as a sign corroborating this view.

2. Section of Bacteriology

Jensen, K. A. and Inger Kiær: Problems concerning the estimation of the chemosensitivity of microbes and measuring of penicillin and streptomycin concentration in the blood and spinal fluid

146

Zetterberg, Bo: Effect of some new substances on the metabolism and morphology of tubercle bacilli.

The substances here examined are: methylindol, methyluapathalenes and methylquinolines, besides compounds of these and sulfanilamides. Their bacteriostatic, bactericidal and respiration-inhibitory properties are described, together with their influence on the tuberculin production and morphology of the tubercle bacilli.

Discussion.

DAVIDE, H.: In connection with the demonstration of his particularly interesting experiments, Dr. Zetterberg showed also a Warburg curve on which the tubercle bacillus control presented a typical respiratory curve.

Now I should like to ask Dr. Zetterberg whether his T. B. strain did not often show a poor respiration or practically no respiration at all. In the great number of Warburg experiments I have carried out together with Theorell and Bergström, employing several antibiotic factors active against tubercle bacilli, a good many experiments turned out unsuccessful because of poor oxygen absorption by the bacilli. Tubercle bacilli belonging to the same strain, and being of same age (as a rule 14 days) and grown on the same medium were found to vary in an extraordinarily high degree as to their rate of respiration. It was not a question of bacteria that somehow had been injured. On cultivation of the bacilli in human blood on cover slips ad modum Pryce-Mueller I was able through daily microscopic examination to demonstrate that their multiplication rate from the second to the 9th day was equally pronounced in bacilli with normal respiration and in the bacilli which showed no oxygen consumption.

I wish to call attention to these findings even though to me they appear somewhat to lower the value of spirometric assays of the antibacterial activity of the given preparations.

SIEVERS, O.: In connection with Professor Davide's remarks I should like to ask Dr. Zetterberg whether he had noticed any difference in the respiration of the bacteria due to the point of time for the addition of the new substances to the bacterial emulsions in the respiratory experiments.

- Olin, G. and A. Lithander: Toxin-forming staphylococci as cause of death on the injection of infected bacteriological preparations 152
- Gard, Sven: Egg products as source of infection in paratyphoid.

In 1946 a considerable number of cases of paratyphoid infection occurred in man brought about by *Salmonella* types not previously observed here in Sweden. The most likely source of the infection was found to consist in egg products which proved to a large extent to be contaminated.

- Borgen, L. O.: Infection with *Actinomyces muris ratti* after a rat bite 161
- Zettergren, L.: Pulmonary moniliasis.

Preliminary results of animal experiments aimed to show the cases in the lungs (disseminated foci of granulomas) which arise from inhalation of monilia-containing threshing dust.

- Lindau, A.: How are we to reveal an unspecific positive Wassermann reaction?

Survey of serological reactions for syphilis against the data on morbid conditions given by the patients themselves. Discussion about how the false positive reactions may be recognized and followed.

Discussion.

SIEVERS, O.: It is of the greatest importance at every suspicion about an unspecific Wassermann to try to ascertain the cause of the faulty reaction. In order to elucidate this I shall try to give an account of explanation of the unspecific Wassermann reaction in primary atypical pneumonia. The reaction was performed as usual with cholesterinized ox heart extract. The usual serum control was performed in duplicate: 1 tube with the same amount of serum as in the tube containing antigen, and 1 tube with twice this amount of serum. The latter, so-called double serum control, in keeping with old-time practice (Sachs, Laubenheimer) has been used as far as possible to illustrate the anticomplementary capacity of serum. If this double amount of serum gives an inhibition, it indicates an all too strong anticomplementary power, and in the presence of antigen such sera may easily give unspecific reactions — even with smaller amounts of serum. Altogether 40 sera from patients with primary atypical pneumonia were examined, and 6 of them were found to react with Wassermann antigen. In two of these cases there was inhibition in the serum control, and hence these sera are absolutely not to be looked upon as Wassermann positive. In such cases we can only record that auto-inhibition is present. The double serum control shows that self-inhibition has occurred in three additional sera. The result thus indicates that these sera should have a distinctly increased anticomplementary power. Indeed, this could be confirmed through titration. It was found that all the sera reacting with the Wassermann antigen had a greatly increased anticomplementary action. These titration results also suggest that a possible Wassermann positivity in primary atypical pneumonia often — in my material, so far, always — will be due to an increase in the anticomplementary capacity of the serum. By paying close attention to this we may find a cause of an apparently unexplainable reaction.

Something similar has been observed on examination of three cases of malaria. Also in these cases was the double serum control able to explain the cause of the faulty Wassermann reaction.

PACKALÉN, TH.: The knowledge of the great importance of the lipoids to the outcome of the Wassermann reaction as well as the flocculation test in syphilis makes one wonder whether it might not be possible through removal of the lipoids from sera to eliminate certain factors that have a tendency to produce unspecific reactions for syphilis. I should like to know if Professor Lindau has tried extractions of lipoid in suspect unspecific reactions. For there is another serological reaction — the anti-streptolysin reaction which likewise is highly sensitive to changes in the lipoid content of the serum.

I hope I shall be able even at this congress to give a fairly detailed account of studies on strong unspecific increase in the antistreptolysin titers arising through changes in the cholesterol concentration of serum (or exudate), among others, especially in hepatic sera or exudate and in sera contaminated with certain bacteria. When such sera are lipoid-extracted, for instance, after Blix or after McFarlane — both of which procedures spare the serum proteins from denaturation — the unspecific rise in titer disappears. In contrast hereto, the specific rise in titer — i. e., the rise due to antibody formation — remains rather unaffected by the lipoid extraction.

To me it seems conceivable that also some instances of specific syphilitic reaction might be due to shifts in the lipoid aspects and thus perhaps be made to disappear through lipoid extraction of the serum.

KRAG, P.: In Copenhagen a positive Wassermann is a very rare finding in sera from patients with Weil's disease.

Last spring 27 soldiers were found to give strong Wassermann and Kahn reactions, but strikingly weak, dubious or negative Meinicke reaction. These soldiers were hospitalized for bronchitis, sinusitis or bronchopneumonia. Some of the soldiers presented cold-agglutination, but the Wassermann and agglutination by no means covered each other. P. Wiingaard will report these cases.

In 1941—43 the Meinicke reaction was carried out after both the macro- and the micromethod. Comparison of the results showed that in our hands the micromethod gave no information of value that was not furnished by the macromethod.

Strong unspecific Wassermann reactions disappear more slowly than weak reactions when they appear in lung lesions, it is true, but the really protracted unspecific Wassermann reactions without any lung lesion are usually somewhat weak reactions.

BORGEN, L. O.: Professor Lindau's paper has been most interesting to me, and I wish merely to mention a few experiences from the Wassermann material in the Ullevaal material (Oslo).

Most of the unspecific Wassermann reactions we have seen in our material (about 15—20 per 20,000 reactions a year) come from patients with infiltrations in the lungs. I have never seen a positive Wassermann in Weil's disease. In Norway, in the last 12 years, I have examined about 100 cases of Weil's disease. So Professor Lindau's statement about 40 % unspecific Wassermann reactions does not agree with our experiences in Norway; and in our material an unspecific Wassermann in mononucleosis is exceedingly rare.

In unspecific Wassermann reactions, as a matter of fact, we employ about the same procedure as recommended by Lindau.

For routine examination we employ 4 reactions: Wassermann, Kahn, Meinicke II, and Pallida. Absorption of sera with use of the Pallida reaction — as suggested by Dr. Vogelsang, Norway — has given very good results. Sera with anticomplementary (self-inhibitory) effect involve no problem in practice. On titration of such sera with and without Wassermann antigen it happens but very seldom that the matter is not cleared up.

Now and then we have met with the phenomenon that inactivation of the serum makes it give a strong unspecific Wassermann reaction, while the same serum without inactivation — after standing a few days in icebox (for removal of the alexin reaction) — gives a negative reaction — as it ought to.

RENKONEN, O.: In the Serobacteriological Institute in Helsingfors, for the verification of positive syphilitic reactions, we have employed the verifying methods given by Kahn, besides Meinicke's reaction. With the verifying methods and the Meinicke reaction the results, on the whole, have been identical and appear to be useful in uncertain cases.

LINDAU, ARVID: I agree with Dr. Sievers that there is some relationship between anticomplementary effect and unspecific Wassermann reaction.

Unfortunately no really good method for elimination of this anticomplementary effect is yet available; and the same seems to apply to attempts to free the sera from the factors giving unspecific reactions. If the sera are left standing in ice-box for some days, however, we may find that an unspecific Kahn reaction has disappeared in the meantime.

It seems not unlikely that the changes in syphilitic serum are of quantitative rather than qualitative nature. This makes it easier to understand the appearance of the unspecific reactions.

- Vogelsang, Th. M.*: Bordet-Wassermann's reaction carried out with cardiolipin-lecithin-cholesterol antigen 167
- Krag, Peter*: The lecture gave facts from three papers:
- M. Bjørneboe and P. Krag*: Studies on a complement fixation in hepatitis with liver extract as the antigen, published in *acta path. et microbiol.* XXIV, 352, 1947.
- M. Bjørneboe, P. Krag and F. Lundquist*: Complement fixation in hepatitis with some lipoids from liver-tissue ... 175
- M. Jersild and P. Krag*: Experiments on transmission of infectious hepatitis to guinea-pigs, to be published in *acta pathol. et microbiol. scand.*
- Scheibel, Inga and Knud Bojlén*: Combined diphtheria and tetanus immunisation 177
- Ouchterlong, Örjan*: In vitro method for testing the toxin-producing capacity of diphtheria bacteria 186
- Ringertz, N. and C.-A. Adamson*: The lymphadenitis picture in man with various types of bacterial invasion 192
- Renkonen, Osv.*: On the constancy and heredity of serological quantities, to be published in *acta path. et microbiol. scand.*
- Westin, G. and J. Wærhaug*: Pathogenesis and pathology of odontolithiasis. No abstract received.

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HEREDITY IN CANCER. CLINICAL AND EXPERIMENTAL INVESTIGATIONS.

By Tage Kemp.

For centuries it has been known that cancer often occurs familiarly; the genetical aspects of the cancer problem have been the object of extensive systematic studies in human beings and experimental genetic investigations on animals, especially on mice. But the problem of the genetic origin of tumors has not yet been definitely solved.

This is the reason why a group of collaborators at the University Institute for Human Genetics in Copenhagen about 5 years ago decided to make an attempt to investigate the genetics of cancer. The investigation has partly been carried out in cooperation with the Danish Cancer Registry, the University Institute of Pathological Anatomy and the Radium Center in Copenhagen and numerous Danish hospitals and clinics.

The investigations so far carried out include

- 1) medical-genealogical studies made by a number of physicians each being a specialist in the field his investigation concerns, and
- 2) genetic experiments in mice.

The statistical-genealogical investigations have been performed as a series of surveys each concerning a considerable number, generally several hundreds, of *proband*s or *propositi* with cancer of a certain type, f.i. cancer mammae, cancer uteri, cancer oesophagi, multiple cancer or leukemia, picked at random from the population. The families have been studied thoroughly and so far as possible every case of cancer in the relatives has been verified; in that respect these investigations differ from earlier more comprehensive statistical studies in the heredity of cancer (*C. Little* (1923), *G. Waaler* (1931), *E. Hanhart* (1943)) and some which partly may be characterised as »proband investigations« (*W. R. Williams* (1908), *E. Lane-Claypon* (1926), *I. M. Wainwright* (1931), *W. Wassink* (1935) and *R. Martynova* (1937)).

So far the investigations of *Oluf Jacobsen* on heredity in breast cancer (1946) and of *Aage Videbæk* on heredity in human leukemia and its relation to cancer (1947) have been published. *O. Brobeck's* study on the heredity in cancer uteri, *E. Mogensen's* study on cancer oesophagi and *O. Feilbergs* study on multiple cancer are under preparation.

Jacobsen's study on the heredity in breast cancer includes 200 probands, 197 women and 3 men with cancer of the breast. The investigations of the families of the probands comprise the following categories of relations: parents, brothers and sisters, grandparents and brothers and sisters of the parents. Various forms of cancer for example lip and skin cancer are not represented in the families of the probands, but cancer of most other forms, sites and types occurs in the relatives and is comprised under the collective term endogenous cancer as a whole.

A study of the case histories of the probands gives no grounds for supposing that exogenous factors play any important rôle in the development of breast cancer. An excess incidence of breast cancer among the female relations of the probands and likewise a significant excess incidence of endogenous cancer as a whole in all the categories of relatives both male and female were found. This indicates hereditary predisposition as the chief factor in the development of breast cancer.

Breast cancer is dependent on hereditary factors and the tendency to this particular form of the disease is bound up with an inherited predisposition to endogenous cancer in general. The development of the endogenous cancers is probably due to a general hereditary predisposition and the localisation of the tumor is determined by endogenous or exogenous factors. The general hereditary predisposition is often apparently inherited as a dominant character.

Videbæk has investigated pedigrees of 209 patients with leukemia and found the familial incidence of the disease to be at least 8 per cent. Several types of leukemia may occur in the same family. The relative frequency with which they occur is the same for the familial cases and for the disease in general. The multiple occurrence in the individual family is usually not confined to some particular type; leukemia is probably genetically a morbid entity.

Leukemia as such is not inherited; it is a question of inherited disposition to the disease. Simple dominance or recessivity may be excluded; it may possibly be a question of failing dominance or polymeria (homologous polymeric factors).

Hereditary diseases are generally monomeric. Predispositions to diseases that in greater or smaller degree are hereditary must generally be regarded as polymeric. They are graded characters dependent on many genes, which appearing alone have but little effect, and of course furthermore dependent on the gene milieu of the whole organism and the environments.

Among members of a family there seems to be an age correlation as regards the onset of leukemia.

The incidence of pernicious anemia is significantly higher among the relatives of patients with leukemia than among the relatives of the control probands. The relation is probably due to a common hereditary predisposition.

Among the relatives of the leukemia patients there is a significantly excessive incidence of cancer as a whole, due to the high incidence of all forms of cancer. Cancer including leukemia is probably a disease entity genetically dependent on a dominant gene common for all the different forms of endogenous cancer.

The development of leukemia seems to depend on various conditions, among others, on non-specific hereditary predisposition to cancer, which is believed to be present in at least 20 per cent of the population in general, and partly on one or several genes, the activity of which plays a rôle for the localisation of the cancer to the leukon. Leukemia seems to constitute an entity genetically and changes in the circumstances of the individual, the environments taken in the widest sense, are determining for the type of the disease (acute or chronic lymphogenous or myelogenous, monocytic or stem-cell leukemia) which develops. External factors play a rôle for the development of leukemia.

The probability of the occurrence of several cases of leukemia in the same family is rather slight; the risk of near relatives of a patient with leukemia getting cancer is as high as up to fifty per cent.

Brobeck investigated the families of patients suffering from cancer uteri. Among the relatives of probands with cancer corporis uteri the incidence of endogenous cancer was high, and the relatives of probands with cancer colli uteri show the same frequency as the relations of control probands. In the families of the patients with cancer colli uteri, on the other hand, a relatively increased frequency of cancer oesophagi was found; in this connection it is worth while to remember the similarity with regard to the histological structure of cancer colli uteri and cancer oesophagi.

In the families of probands suffering from cancer oesophagi investigated by *Mogensen* an increased number of cancer oesophagi was found. Generally the second case of cancer oesophagi in the family was found in a close male relative, the father or a brother of the proband. In these families, however, the proband and the relative with cancer oesophagi were as a rule both of them suffering from chronic alcoholism; probably it was the tendency to alcoholism and not the tendency for cancer, which was inherited, the alcoholism being the cause of c. oesophagi.

Feilberg investigated the families of probands with multiple primary cancer; all of them had besides breast cancer some other malignant tumor. The relative frequency of cancer in the relatives of

the probands was almost equal to the frequency in the relatives of patients with cancer mammae; the incidence of cancer corporis uteri seems, however, to be relatively increased compared with the frequency of cancer colli uteri, in the families of probands with multiple cancer.

Julius Bauer (in a Middleton Goldsmith Lecture, New York Pathological Society October 12, 1932) supposed in accordance with Loeb & Lathrop, Waaler, Miche *et al.* that the constitutional tendency to cancer is based on the effect of two predisposing genetic factors: a general blastoma tendency and a localisation factor; but both genetic factors may in exceptional cases, to a certain extent, be replaced by excessive, exogenous irritation, or they can be induced by exogenous injuries.

In the classic experimental animal for tumor research, the mouse, the heredity of spontaneous cancer (including leukemia) and of the susceptibility to transplanted cancer have been investigated thoroughly. So far the results obtained have been various, however, and there is a great divergence of opinion with regard to the mode of inheritance (*C. Lynch, M. Slyc, C. Little, N. Dobrovolskaja-Zawadskaja, W. Furth, W. S. Murray, J. J. Bittner, A. M. Cloudman, H. Lefèvre et al.*).

This is why a team of workers in experimental cancer research in the University Institute for Human Genetics in Copenhagen some years ago started experimental studies of the genetics of tumors in mice (*G. Hogreffe, V. Arentsen, W. Permin, N. Betak-Petersen, O. Nygaard-Jespersen and E. Petersen*).

Several mouse strains inbred through many generations have been used in the experiments, one with a high frequency of leukemia, two with a high frequency of cancer mammae and two with a very low frequency of tumor and leukemia. From crossing and back-crossing of these strains result a number of hybrid generations which have been investigated carefully.

The mouse strains with a high frequency of cancer are produced by selection and inbreeding through many generations; therefore it is not proper to compare these strains with human populations. The circumstances with regard to cancer in the hybrid generations obtained from crossing of strains with a high frequency are, however, highly parallel to the circumstances occurring in a human population.

Furthermore the inheritance of the susceptibility to transplantation of malignant tissue has been investigated. Some of the results of the mouse experiments deserve to be mentioned briefly:

The susceptibility to transplanted tumor leukemia tissue is dependent on the presence of one or more dominant genes, characteristic of the tumor line in proportion to the strain of animals employed. The susceptibility is to a certain degree dependent on the presence of one or more dominant genes common to the transplant and the host. When tumor tissue, however, is transplanted to a strain with low frequency through several generations it will accommodate to the strain and the frequency of takes will increase. Hybrid generations of a strain

with a high susceptibility and a strain with a low frequency of takes show the same susceptibility whether the father is from the susceptible the mother from the non-susceptible strain, or conversely.

On the other hand, young ones from a strain not susceptible to transplantation of tumor tissue, when suckled by mothers from a susceptible strain show increased susceptibility, and young ones from a susceptible strain, when suckled by mothers from a not susceptible strain show reduced susceptibility to transplantation of tumor tissue.

A very important observation by Hogrefe is that the pathological-anatomical picture resulting from the growth of a leukemic transplant is also dependent on genetic factors in the host.

Also the studies of the inheritance of spontaneous mammary cancer and leukemia in mice are worth while mentioning: By cross-breeding of a strain with high frequency of breast cancer with an apparently cancer-free strain, the existence of a hereditary predisposition to cancer was confirmed; but furthermore it was shown that a number of factors besides the inherited disposition contribute to the development of mammary cancer. Tumors that occur normally in inbred strains can be accelerated by means of carcinogenic carbohydrates. This acceleration may express itself both by an increase of the tumor incidence and by lowering of the tumor age. Several forms of tumors can be accelerated when there is a hereditary disposition in the strains concerned towards more forms of tumor, and there is an interplay between the genotypical and the external factors.

In mice cancer of the breast is more frequently inherited through the mother than through the father, but this characteristic has not been demonstrated as to leukemia. The investigations on the inheritance of leukemia in mice demonstrate in the filial generations a segregation in early and late cases of leukemia, dependent on genotypic factors. Probably the disease has a tendency to occur later and more seldom in heterozygous than in individuals homozygous with regard to the predisposition for leukemia. This observation is in accordance with the clinical investigations carried out by *Jacobsen* and *Videbæk* mentioned above concerning breast cancer and leukemia in human beings. In either of the diseases the familial incidences seem to manifest themselves earlier than is usually the case with these diseases.

The experiences from both experimental and clinical investigations confirm that the probability of the mammary cancer of the leukemia developing at an earlier period of life is greater, when the genetic conditions are particularly favourable than when there is a demonstrable hereditary taint. On the other hand, the possibility of the occurrence of the disease becomes greater with advancing age even though the mosaic of the genes is less favourable for its development. The development depends in first place on inherited tendency, those with heavier taint become grouped in younger age classes, while the apparently untainted live longer before the growth manifests itself. In genetic term it

may, according to *Jacobsen*, be expressed by saying that the tainted are homozygous as regards one or several genes, whereas the apparently untainted are heterozygous.

The results and conclusions of the aforementioned clinical and genetical investigations on the genetics of cancer may be summarized in the following scheme concerning the genesis of malignant neoplastic growth:

The genesis of malignant neoplastic growth.

Endo- genous Factors	{	<i>Hereditary predisposition</i>	{	Dominance (irregular), recessivity(?) polymeric or multifactor inheritance, homologous polymeric factors. Tu- mors of different forms, types and sites differ in their genetical be- haviour.
		General predisposition		Later and more seldom in hetero- zygous than in homozygous. Variation in manifestation. Variation in susceptibility or re- fractoriness to tumor formation or tumor transplantation. Genes with the character of virus.
		Tendency to localisation (localisation genes, or- gan factors, similar histological structure.)		
Environ- ments	{	Somatic mutation (induced or spontaneous)	{	extrachromosomal
		Cytoplasmic inheritance (maternal ef- fect)		
		<i>Internal milieu</i> (adaptation to individual, type, race or variety and species)		
		Hormonal unbalance, metabolic disturbances	{	
		<i>Modification of internal milieu caused by:</i> transplantation of eggs or embryos, milk factor (maternal in- heritance?), nutrition, age, radiation (decreased resistance), car- cinogenic agents (acceleration), intoxication.		
		<i>External milieu</i> (exogenous factors) Irritation by trauma, chemical, thermal or ray influence, para- sites, bacteria and viruses.		

Summary.

A survey is given of recent clinical and experimental investigations carried out during the last five years in the University Institute for Human Genetics in Copenhagen. A new scheme concerning the genesis of malignant neoplastic growth is presented.

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- in Opera ex domo biologiae hereditariae humanae Universitatis Hafniensis, Vol. 9, 11, 13 and 18.

The investigations mentioned above were carried out with the aid of grants from *Anders Hasselbalchs Fond til Leukæmiens Bekæmpelse*, *Kong Christian X's Fond*, *Landsforeningen til Kræftens Bekæmpelse* and *The Lady Tata Memorial Trust*.

DISCUSSION

J. CLEMMESSEN: I wish first to express my great appreciation of the collaboration between the Danish Cancer Register and the University Institute of Human Genetics, Copenhagen, through a number of years. The original cause of the part of the work due to this collaboration was that in studies on the cancer mortality for various occupations as, for instance, the hotel trade, cancer of the oesophagus was encountered frequently. Now the problem was: Does exogenous cancer arise only in individuals with a hereditary disposition, or at any rate preferably in such individuals?

Presumably this question might be settled through simultaneous investigations on exogenous as well as endogenous forms of cancer in a population worked up statistically.

The possibility of establishing the inheritance seemed rather slight, however, as relations more distant than sibs and parents are very difficult to plot because the information obtained is not reliable.

T. KEMP: I agree with Dr. Clemmesen as regards the value of a registration of all known cases of cancer in a population, carried out during a considerable number of years. A cancer registry carefully collected and filed for a long period of years will be useful as a basis for our continued studies on the genetics of tumors. Furthermore the investigations on cancer in twins started some years ago in cooperation by the Cancer Registry and the Institute for Human Genetics may be of use.

THE DANISH CANCER REGISTRY. PROBLEMS AND RESULTS

By *Johannes Clemmesen*.

The Danish Cancer Registry under The National Anti-Cancer League was founded in 1942 with support from The National Health Service in whose offices it works. The purpose of the Registry is the collection of information on the incidence of cancer. All cancer patients admitted in Danish hospitals are voluntarily notified to the registry. A token salary of 1 Krone is paid for each notification and for cancelling a previous one. This material is supplemented with copies of all death certificates for cancer patients which substitute notifications from general practice, and are used for checking up hospital notifications. Autopsies are separately notified.

Investigations following the lines laid down in the paper of Dr. Kemp and the following discussion were the first task of the Registry. It was our intention simultaneously to investigate the extent to which endogenous (i. e. hereditary) and exogenous factors are concerned in the causation of various forms of cancer occurring in a population cross-examined through registration of all cancer cases. These investigations comprise studies on breast and uterine cancer, leukemia and multiple cancers.

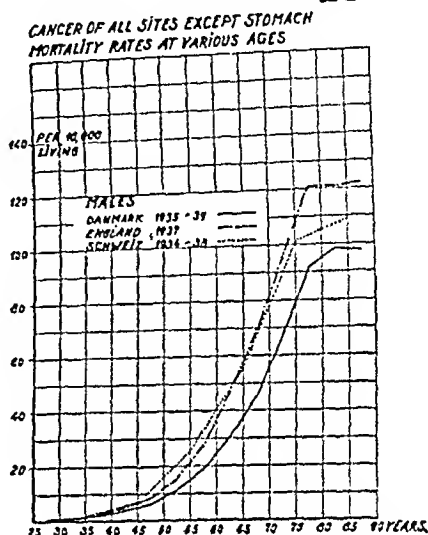
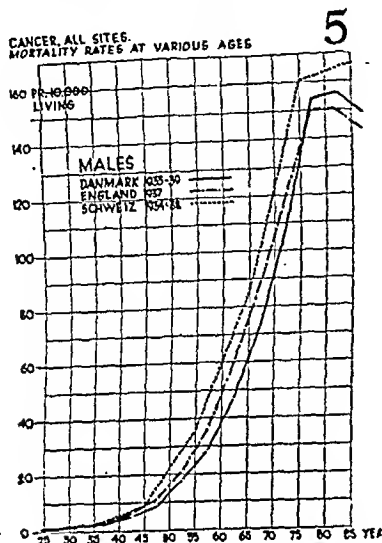
So far, mortality statistics had offered the only basis for international comparisons of cancer incidence. To create possibilities for comparing the results of the Cancer Registry with figures from other countries we have, to begin with, compared age distribution curves for cancer mortalities of 1938 in the three European countries with highest cancer mortalities 1) Switzerland, 2) England and 3) Denmark.

These three countries show only minor differences in total cancer mortality, but Switzerland exhibits an excess for males which show the highest mortality of this sex, while the Swiss female cancer mortality is no. 3. England is second for both sexes, while Denmark is first in female cancer mortality and no. 6 with regard to male cancer. Which factors are responsible for these differences?

Table 5 gives the total cancer mortalities at various ages for males in the three countries. The corresponding diagram for females show smaller deviations in opposite directions.

If gastric cancer is excluded from the total cancer mortality for all sites we find that the curves for the two countries of highest mortality coincide, as shown in Table 15 (Switzerland and England). The corresponding curves for women display the same feature: the two

15



upper curves (here for Denmark and England) coincide when gastric cancer is subtracted from the total.

If cancers are divided into two groups, one with all cancers of accessible sites, and the other with cancers of inaccessible and uncertain sites, then the elimination of gastric cancer from »inaccessible sites« will cause coincidence of all three curves for females, but not for males (Tables 12 and 13). Computations have confirmed the impressions given by the diagrams.

Thus the group termed gastric cancer is to a very large extent responsible for the differences in cancer mortality between the three countries concerned. However ill-defined that group may be, it is also responsible for the differences in female inaccessible cancers.

Similarly in a Danish material where Capital, Provincial Towns and Rural Areas take the places of the three countries, female inaccessible cancers will show coincidence of the three curves after the elimination of gastric cancer. The impressions given by the diagrams have all been confirmed by elaborate computations worked out by Th. Busk, Lic. aet.

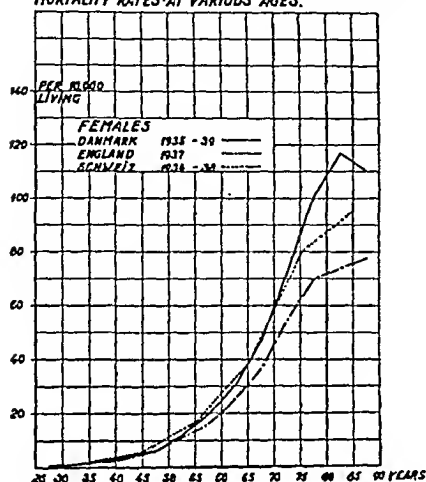
However, the main object of The Cancer Registry in Denmark is comparison of figures which are nearer to the real incidence of cancer, than mortality figures.

We may now compare the figures from The Danish Cancer Registry with those of the Cancer Registration for Upstate New-York (exclusive of New York City). The figures are given as percentage of all cancer,

and it should be noticed than for final conclusions age distribution curves should be constructed. The following figures will however, do for an orientation.

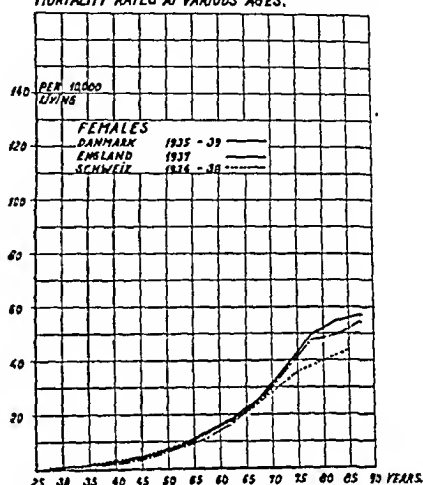
12

CANCER OF INACCESSIBLE SITES, INCLUDING STOMACH
MORTALITY RATES AT VARIOUS AGES.



13

CANCER OF INACCESSIBLE SITES, STOMACH EXCLUDED
MORTALITY RATES AT VARIOUS AGES.



Material of The Danish Cancer Registry 1942—1944 compared with Material from New York State, exclusive of New York City, 1943.

The difference in the incidence of skin cancer is easily explained by the difference in latitude of the two states. The differences in gastric

Males				Females			
Denmark		New York State		Denmark		New York State	
Stomach	23%	Skin	14%	Breast	20 %	Breast	23 %
Rectum	10%	Prostate	11%	Uterus	18 %	Uterus	18 %
Skin	9%	Stomach	10%	Collum uteri	11,9%		10,0%
Intestine	9%	Intestine	9%	Corpus uteri	3,9%		3,4%
Prostate	6%	Bladder	6%	Unspecified	1,7%		4,4%
Lung	5%	Rectum	6%	Stomach	16 %	Intestine	9 %
Esophagus	4%	Lung	4%	Intestine	8 %	Skin	8 %
				Skin	6 %	Stomach	6 %
				Rectum	6 %	Ovary	5 %
				Ovary	5 %	Rectum	4 %

The total includes Leukosis and Lymphogranulomatosis Hodgkin.

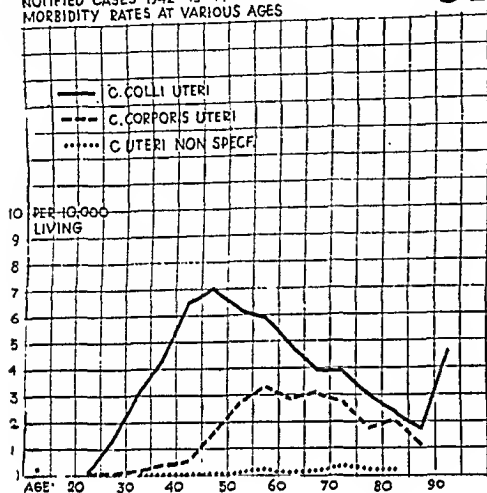
cancer correspond to those previously found in mortality statistics, and suggest that these differences are real, although the cause is still obscure. The differences in the percentage of prostatic cancer might to some extent be attributed to differences in histological traditions. This problem is the subject of special studies by Dr. H. Starklint.

The female cancers display a surprising similarity between the two states, also with regard to the incidence of the two forms of uterine

cancer. In comparing these figures it should be remembered that the Danish material shows fewer cases of uncertain site than the American. It is to be hoped that comparisons with due regard to the possible differences in age distribution of the population at risk can be carried out in collaboration with the Cancer Registration offices of New York State.

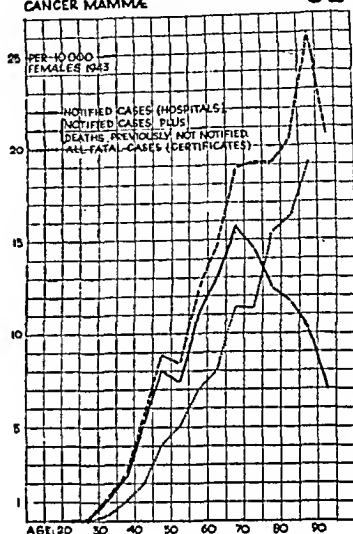
THE DANISH CANCER REGISTRY
CANCER UTERI
NOTIFIED CASES 1942-43-44
MORBIDITY RATES AT VARIOUS AGES

51



THE DANISH CANCER REGISTRY 1942-43-44
CANCER MAMMÆ

52



In his work on heredity in breast cancer, Oluf Jacobsen found an uneven age distribution of mammary cancer. Table 52 gives the age distribution curves both of the mortality figures, computed by means of the death certificates alone, and of hospital cases alone, as well as of hospital cases supplemented with death certificates for patients never admitted in hospital. The latter group makes about 12 per cent of the total.

The most outstanding feature of the diagram is the decrease in incidence of breast cancer between the ages of 45 and 55, a feature which confirms Jacobsens observation as valid for the entire Danish material. We would suggest that the reason for this observation not being made earlier is that most materials are subdivided in age groups of ten years, and perhaps that age may previously have been less accurately stated. (Here the age for the onset of the disease is given as age at first admission in hospital. For death certificates age is given as age at death.) It is tempting to suppose that the decrease in incidence of breast cancer has some connection with the age for the cessation of the menstruation.

Furthermore it is evident from the diagram that this material, as all other statistical materials bearing on cancer in a whole population is suffering from falling in two groups: hospital patients and cases treated privately. However, this does not apply to the Danish material

of specified uterine cancers, given in Table 51, of which more than 90 per cent have been treated in hospitals.

This diagram clearly explains why some countries must show an increasing percentage of corpus cancer. With the curve for cervical cancer showing a climax, an average aging of the population at risk must cause an increasing fraction of corpus cancer.

Finally the author reported studies on the incidence of lung cancer, the mortality from which for males in Copenhagen has been tripled from 1931 to 1945. The figures for females showed a slight rise for the same period, and consequently the sex proportion had changed from 1: 1 in 1931 to 3 males to 1 female in 1945.

Analyses of the material from The Central Tuberculosis Station in Copenhagen showed that an unselected material of lung patients referred by general practitioners, which during the years 1935—1945 had been examined with more or less the same diagnostic technique, exhibited a constant sex proportion of 1 female to about 8 male cases of lung cancer. There was no increase of lung cancer in males corresponding to the rise in mortality figures either in constancy or in height.

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DISCUSSION

F. HENSCHEN: Doktor *Clemmesens* Vortrag veranlasst mich, einen kurzen Bericht über die Sterblichkeit an Karzinom in Stockholm während der letzten Jahre zu geben. Meine Angaben gründen sich einerseits auf die Zahlen der öffentlichen Statistik der Stadt, andererseits auf das Sektionsmaterial des Karolinischen Instituts 1896—1939 und des Krankenhauses St. Erik 1925—46. Die stark steigende Lebensdauer der Bevölkerung und der grosse Überschuss an Frauen, besonders in den höheren Lebensaltern, beeinflussen in hohem Grade die absoluten Zahlen; es sind deshalb eigentlich nur die relativen Zahlen, die in diesem Zusammenhang von Interesse sind.

Die Sterblichkeit an Karzinom, sämtliche Formen, ist nach der offiziellen Statistik während der letzten 40 Jahren stark gestiegen und zwar bei Männern von 9,8 bis 15,4 und bei Frauen von 14 bis 15,4 pro 10,000 (Durchschnittszahlen von Fünfjahresperioden). Die beiden Geschlechter zeigen also jetzt dieselbe Mortalität an Karzinom.

Die Sterblichkeit an *Cancer mammae* stieg während dieser Periode von 1,5 bis 2,8. Die Sterblichkeit an *Cancer ovarii et uteri*, die allerdings nur während der letzten 20 Jahre registriert worden ist, zeigte eine Steigerung von 3 bis 3,4. Die Sterblichkeit an *Cancer prostatae* stieg während derselben Periode von 1,1 bis 1,8, also um etwa 75 %. Auch in diesen Fällen handelte sich um Durchschnittszahlen von Fünfjahresperioden.

Die *Karzinome des Digestionskanals* zeigen folgende, z. T. abweichende Verhältnisse: Die Sterblichkeit an Mund-, Zungen-, Schlund- und Speiseröhrenkrebs sank während der letzten 10 Jahre bei Männern von 1,6 auf 1,5 pro 10,000. Bei Frauen hielt sie sich während der ganzen Periode bei 0,5. Die Sterblichkeit der Männer ist also hier dreimal so gross wie die der Frauen. Die Sterblichkeit an *Magenkrebs* ist im Laufe der letzten 40 Jahre (1906—45), in scharfen Gegensatz zu der übrigen Kancermortalität, deutlich gesunken, und zwar bei Männern von 5,5 bis 4,3 und bei Frauen von 4,2 bis 3,4 pro 10,000 (Durchschnittszahlen wie vorher). Die Männer sind durch diesen starken Niedergang ihrer Sterblichkeit den Frauen näher gekommen; während des letzten bisher registrierten Jahres (1945) lagen die Zahlen der beiden Geschlechter so niedrig wie 4,0, bzw. 3,5. Die absolute Zahl der Todesfälle an Magenkrebs lag zum ersten Mal höher bei Frauen als bei Männern (126 Frauen, 118 Männer), was durch den starken Frauenüberschuss bedingt ist. Das Sektionsmaterial des Karolinischen Instituts zeigt in den Jahren 1899—1939 ein Sinken des prozentuellen Anteils der Karzinome im oberen Teil des Digestionskanals (Lippen bis Pylorus mit einbegriffen) von 57 auf 33 % der gesamten Karzinome. Der Anteil der Magenkrebsse allein ging von 52 auf 17 % zurück (letzte Ziffer ist jedoch durch Zufall allzu niedrig). Ähnliche Zahlen erhielt ich aus dem Sektionsmaterial des Krankenhauses St. Erik, wo die Fälle von Magenkrebs an fünf nacheinander folgenden Gruppen von 3000 Sektionen berechnet wurden. In den ersten Jahren nach 1925 bildeten sie 80 % aller Krebsfälle, aber in den letzten Jahren, einschliesslich 1946, nur 41 %. Die offizielle Statistik der Stadt zeigt ein Sinken der Sterblichkeit an Magenkrebs von etwa 43 % (1911—15) auf kaum 25 % (1941—45) der gesamten Krebssterblichkeit. Im Gegensatz zum Magenkrebs weist der *Darmkrebs* (Kolon und Rektum) eine nicht geringe Steigerung auf, und zwar in der offiziellen Statistik von 1,4 bis 3,1 bei Männern und von 1,2 bis 2,4 bei Frauen. Im Sektionsmaterial des Karolinischen Institute stieg der Anteil dieser Krebse von 8 auf 16 % aller Krebse und im Sektionsmaterial des Krankenhauses St. Erik von 20 bis 42 % der Krebsfälle. Die offizielle Statistik zeigte eine prozentuelle Steigerung von 11 % auf gut 15 % der Todesfälle an Krebs.

Es dürfte berechtigt sein, folgende Schlüsse zu ziehen: Die untersuchten Formen von Krebs zeigen, mit Ausnahme der Krebse des oberen Teiles des Digestionskanals, durchgehend eine mehr oder weniger starke Steigerung der Mortalitätsziffern, die indessen vor allem durch

die Schnell steigende mittlere Lebensdauer und die verbesserte Diagnostik zu erklären ist. Ob eine wirkliche Frequenzsteigerung in gewissen Fällen vorliegt, muss weiter untersucht werden.

In scharfen Gegensatz dazu zeigt die Sterblichkeit an Karzinom im oberen Teil des Digestionskanals, einschliesslich Pylorus, und vor allem an Magenkarzinom, eine deutliche Verminderung, und zwar sowohl in der offiziellen Statistik, als auch im Sektionsmaterial zweier Prosekturen.

Die Ursache dieser Verminderung der Mortalität an Magenkarzinom ist meiner Meinung nach darin zu suchen, dass die Grundkrankheiten, auf deren Boden sich der Magenkrebs vor allem entwickelt, also in erster Linie die Gastritiden, weniger frequent geworden sind, was ich mit der erheblichen Verbesserung der sozial-hygienischen Verhältnisse während der letzten 40 Jahre in Verbindung setzen möchte. Die Bevölkerung Stockholms ist sozusagen in eine höhere soziale Klasse aufgestiegen.

Es ist von Interesse, diese Stockholmer Verhältnisse mit den von *Cramer* in Bayern und von *Stevensen* in England nachgewiesenen zu vergleichen. In diesen beiden Ländern zeigte die Frequenz des Magenkrebses einen auffallenden Parallelismus mit dem Beruf und den sozialen Standard: je höher der Standard, um so weniger Magenkrebs, bzw. Krebs des oberen Teils des Digestionskanals. Unterhalb des Pylorus bestand nach *Stevensen* kein deutlicher Unterschied in der Kanzerfrequenz. In derselben Weise dürfte die stärkere Senkung der Frequenz des Magenkrebses in Stockholm beim Mann als bei der Frau zu erklären sein: das »Ventrikelmilieu« der Männer hat sich offenbar schneller verbessert als dasjenige der Frauen.

P. S. Nach dieser Aussprache ist eine Untersuchung von *Clemmesen* über »Cancer ventriculi in den verschiedenen Ländern« zu meiner Kenntnis gekommen. *Clemmesen* konnte auch in Dänemark einen ähnlichen, scheinbaren Rückgang der Sterblichkeit an Magenkarzinom feststellen. Die Senkung der Frequenz war in Köbenhavn, wie in Stockholm, viel deutlicher bei Männern als bei Frauen. Die Zahlen waren ferner durchgehend am niedrigsten in Köbenhavn, höher in den Provinzstädten und am höchsten in den Landdistrikten. *Clemmesen* meint, dass sowohl diese auffallende deutliche allgemeine Senkung der Zahlen, als auch die grossen Unterschiede zwischen Köbenhavn, Provinzstädten und Land, vor allem durch die fortschreitende Verbesserung der Diagnostik zu erklären seien. Geographische Unterschiede liessen sich jedoch nicht ausschliessen. In der Tat hätte man, seiner Meinung nach, Magenkrebs früher allzu häufig diagnostiziert, vor allem auf dem Lande, wo die diagnostischen Hilfsmittel am geringsten wären.

Die dänischen Zahlen stützen sich, wie *Clemmesen* bemerkt, auf Berichte der Krankenhäuser und auf Todesattesten, welche oft Patienten betreffen, die zu Hause starben. Die Stockholmer Zahlen ent-

stammen, wie angeführt, teils der öffentlichen Statistik, und sind also in dieser Hinsicht mit dem dänischen vergleichbar, teils aber dem Sektionsmaterial zweier Prosekturen, wo die verbesserte Diagnostik keine Rolle spielen kann. Und wie soll man erklären, dass der Magenkrebs allein sinkende Zahlen zeigt, während die Frequenz aller anderer Krebsformen steigt? Auch bei diesen macht sich ja die bessere Diagnostik merkbar. Und weshalb sinkt die Frequenz des Magenkrebses schneller beim Mann als bei der Frau? Ich bin aus diesen Gründen geneigt, die von *Clemmesen* gefundenen, an sich sehr interessanten Frequenzveränderungen und Frequenzunterschiede eher durch geographische und sozial-hygienische Faktoren als durch die verbesserte Diagnostik zu erklären.

E. ASK-UPMARK: Concerning the decrease in the frequency of cancer of the stomach in Stockholm as reported by Professor Henschen, one naturally wonders whether the cases of pernicious anemia might not play a role in this respect. Before the introduction of the specific treatment in this lesion the gastritis would take its usual course, and the disturbances in the mucous membrane were most likely favorable to the development of cancer, which in such cases was found to be strikingly common. After adoption of a rational therapy in pernicious anemia, presumably, the conditions for the development of cancer of the stomach are no longer the same as before.

J. CLEMMESSEN: The results reported by Professor Henschen are particularly interesting because cancer of the uterus as well as cancer of the breast is increasing in frequency. Most often cancer of the uterus increases with the fertility, while the incidence of cancer of the breast decreases.

Even though social conditions might play a decisive role in the decrease of cancer of the stomach, this could hardly be the reason why Great Britain showed a lower mortality for this disease than Switzerland and Denmark.

As to the value of autopsy statistics, the autopsy percentages for various forms of cancer, for the entire Denmark, in 1943—44 were as follows:

Cancer of the stomach	14 %
» » » prostate	24 %
» » » lung	47 %
» » » oesophagus	25 %
» » » breast	18 %
» » » cervix & uterus	30 %

Undoubtedly the differences in these values are due in part to differences in hospitalization; at any rate they illustrate the difficulty in arriving at any conclusion as to the frequency of cancer in a population from the autopsy frequency of that disease.

COMPARISON OF THE EPIDERMAL HYPERPLASIA IN THE SKIN OF MICE AFTER APPLICATION OF CARCINOGENIC AND NONCARCINOGENIC IRRITANTS

By N. O. Berg.

In the previous lecture by Ahlström and Berg it was emphasized that the cytoplasm of the epidermal cells of mice after application of benzpyrene in a 0.5 % solution to begin with presented diffuse fluorescence which, from 24 to 48 hours after application, perinuclearly was distinctly stronger. This accumulation of benzpyrene or of a fluorescent derivative of it was due to an increase and perinuclear accumulation of cytoplasmic structures rich in lipoids, especially mitochondrias. The reaction was regularly observed on the skin of the back of mice, but neither on their feet, tails and ears, these regions all being less sensitive to the tumour-producing effect of the carcinogenic hydrocarbons. It was likewise distinct in rabbits after application of tar and 9-10-dimethyl-1-2-benzanthracenes, but it could not be elicited in rats or guinea-pigs.

The question now arose whether this skin reaction merely was a sign of hyperplasia of the epithelial cells *per se* or whether it was specific of the carcinogenic hydrocarbons (I). As the reaction subsequently proved not to be specific, it remained to be examined whether the skin reaction to benzpyrene showed any special characteristics in other respects or not (II).

I. *Is the power of perinuclear accumulation of benzpyrene specific of the hyperplasia which is occasioned by the action of carcinogenic hydrocarbons?*

A series of mice (strain Dobrovalskaia-Zawadskaja from the University Institute for Human Genetics, Copenhagen) was exposed to different skin irritants applied on clipped areas on the back of the animals. During the subsequent days a couple of mice were killed every day or every other day. Some minutes before the areas were brushed

with a 0.5 % benzpyrene solution in acetone. The benzpyrene (= Bp) rapidly penetrated into the skin and was deposited in lipoid-containing cell structures. The benzpyrene thus served as *fluorochrome* and indicated the distribution of lipoids in the tissue.

The following skin irritations were used: Turpentine, cantharidin, trichloroacetic acid, and croton oil as well as mechanical trauma.

Results: In the fluorescence microscope the normal skin epithelium impregnated with Bp appears in the following manner: The cells are small, the cell nuclei, appearing as non-fluorescent dark

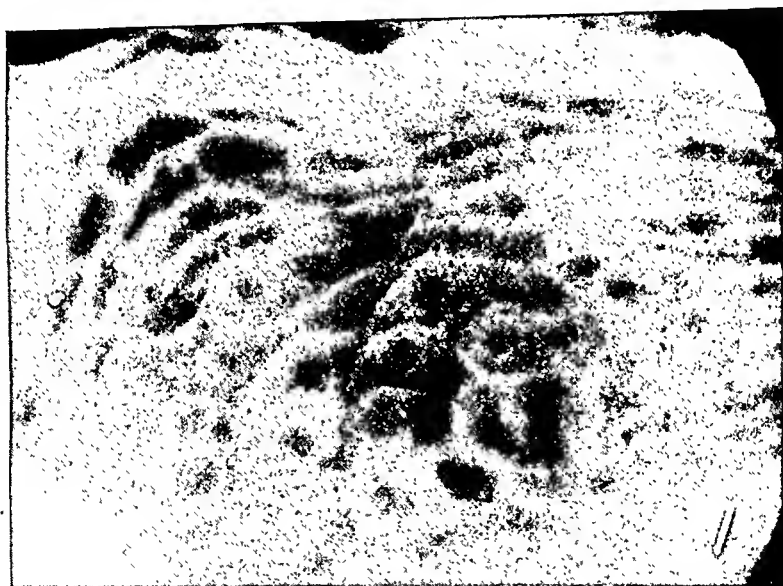


Fig. 1.

Fluorescence of epidermis 3 days after a single application of benzpyrene.

spots, are small too, and the cytoplasm shows diffuse fluorescence. Fig. 1 shows the mouse skin as it appears 2—4 days after the application of a carcinogenic hydrocarbon such as Bp: The nuclear spaces are larger and encircled by strongly fluorescent rings in which there are sometimes seen granules. Outside those rings there is a zone with weaker fluorescence, and in the periphery the cell boundaries often are strongly fluorescent.

After brushing with undiluted *turpentine* a similar picture (Fig. 2) is seen during the first two days. After the lapse of three days, however, the perinuclear fluorescence has decreased considerably — and so has the size of cells and cell nuclei.

During *wound-healing*, epidermal hyperplasia is seen both in the

very border of the wound, and a little peripherally to it. From 3 to 9 days after the scarification of the skin there are seen distinct »rings« round the nuclei in the large cells of the hyperplastic skin area.

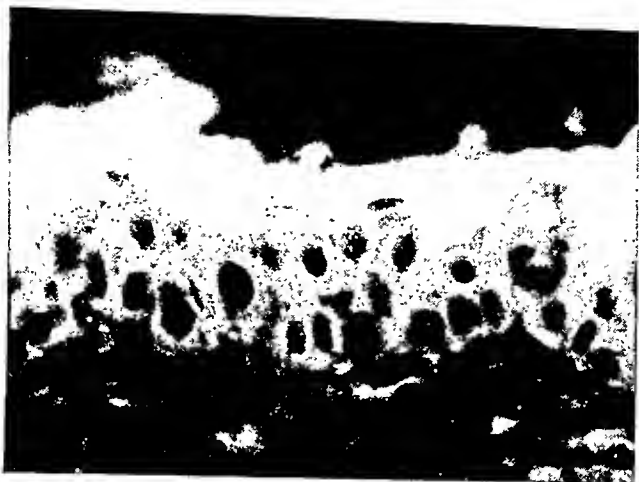


Fig. 2.
Fluorescence one day after application of turpentine.

Substances, which destroy the skin epithelium, such as *cantharidin* in a 0.5 % solution, and *trichloroacetic acid* in a 10 % solution in acetone may give a similar picture. Application of these solutions gives complete destruction of the epithelium. After the lapse of from 4 to 6 days, however, a regeneration of the epithelium ensues from the hair follicles and from the skin epithelium surrounding the treated area. After a week the greater part of the area is covered by a high epithelium which shows large cells with pronounced condensation of the fluorochrome around the nuclei (Fig. 3).

A 0.5 % solution of *croton oil* brings about a corresponding picture. Here the epithelial injury is slight, however, and the hyperplasia appears already during the first 24 hours. At the same time weak fluorescent rings appear around the nuclei. These rings increase in density during the subsequent two days, being most pronounced after 3—5 days.

As in the previous examinations there was a close parallel between the amount of mitochondria and the strength of the perinuclear condensation of Bp: a pronounced perinuclear condensation was always associated with numerous mitochondria. As is evident from Fig. 4, they were accumulated around the nuclear membrane, often somewhat asymmetrically, sometimes in polar arrangement around the elongated basal nuclei. I have not seen any perinuclear condensation or increase of mitochondria in the epithelium not presenting hyperplasia.

Summing up this part of the investigation has shown that a peri-

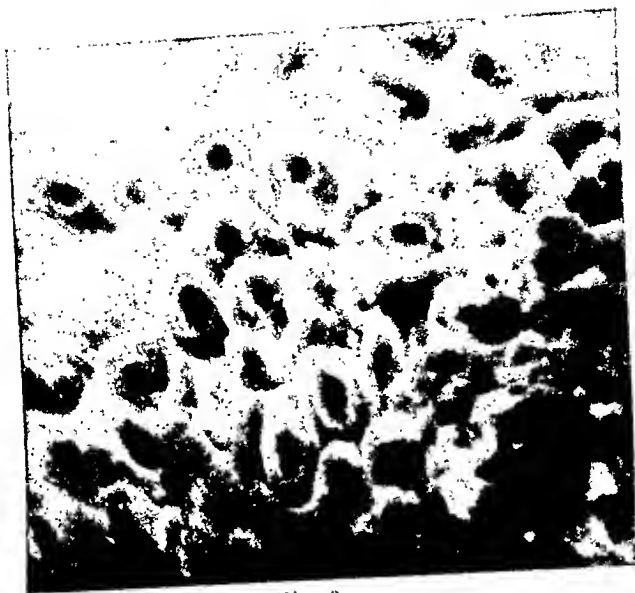


Fig. 3 a.



Fig. 3 b.

Fluorescence 7 days after application of cantharidin (0,5% solution in acetone). a) stratum spinosum. b) stratum basale.

nuclear accumulation of lipoid and an increase of mitochondria ensues during the development of any epidermal hyperplasia regardless of its being caused by carcinogenic or unspecific irritants. This result agrees with the result of the previous investigations: the reaction appeared only in those animals and within those areas, where the carcinogenic substances gave rise to epithelial hyperplasia. If the epithelial hyperplasia fails to appear, there does not either appear any mitochondria reaction.

II. *Has the epithelial hyperplasia appearing after application of carcinogenic hydrocarbons any specific characteristics or not?*

The majority of investigators, particularly Pullinger, Cowdry and co-workers as well as Glücksmann, have emphasized that certain features were characteristic or possibly specific of the carcinogenic hyperplasias on the whole, namely,

(1) The discrepancy between the slight initial cell lesion and the strong hyperplasia.

(2) Disturbances of mitosis during the first 24 hours.

(3) The great increase of the volume of the cells as well as the cell nuclei.

The aim of my investigation is in that respect to compare the epithelial hyperplasia after application of benzpyrene with the hyperplasia produced by application of the above-named irritants.

The degree of the primary cell lesion and the relation to the subsequent regenerative hyperplasia in the epithelium will merely be mentioned. Certain differences were already referred to. After application of cantharidin and trichloroacetic acid, which give rise to severe cell lesions with necrosis, the hyperplasia does not develop before 5 or 7 days have elapsed. The difference in the mode of development of the epithelial hyperplasia here is obvious. After application of turpentine or diluted croton oil the epithelial lesion is quite insignificant, and the hyperplasia appears already during the first days. Here the deviation from the effect of the application of benzpyrene is less pronounced. Even in this case the primary cell lesion is slight, whereas the epithelial hyperplasia appears somewhat later, namely, after 2—4 days.

Analysis of the mitoses.

In case of turpentine hyperplasia Glücksmann has observed several mitoses, whereas Mottram has seen such in case of croton oil hyperplasia. Moreover, all authors are agreed as to the carcinogenic hydrocarbons causing an increase of mitoses after the third day, opinions differing, however, with regard to the first days. Pullinger as well as Hamperl, Graffi and Langer and others opine that a disturbance of mitosis ensues, with few or no mitoses, whereas Glücksmann thinks that the number of mitoses increases already during the first days. Glücksmann has performed very careful mitosis counts in mice after Bp application to the skin of the back. He, however, has included all the stages of mitosis from prophase to telephase, whereas other researchers more or less clearly declare that it is the number of metaphases which is responsible for their judgment.

I myself have counted a great number of mitoses from the earlier stages of turpentine, croton oil and Bp hyperplasia. The mitoses were



Fig. 4.

Increase of mitochondria 7 days after application of cantharidin.
Mitochondrial staining according to Regaud.

divided into four groups, as shown in Fig. 5, namely, prophase, metaphase — which is again divided into monaster and diaster stages — and telephase mitoses. Fig. 5 shows the percentual distribution of the different mitotic stages within the area of hyperplastic epithelium. It

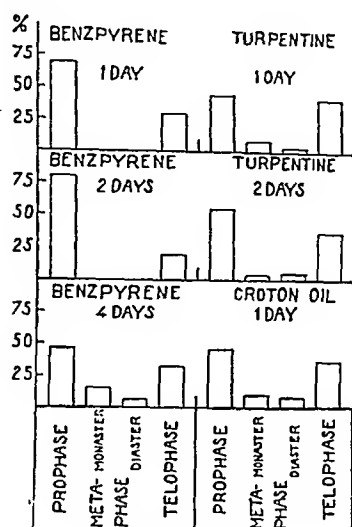


Fig. 5.

Percentual distribution of the mitotic phases.

is evident that there is a disturbance of mitosis in the Bp hyperplasia. This probably is due to an obstruction at the transition to the metaphase.

The figures for the prophase and telephase mitoses unfortunately are not absolutely reliable, because there are a good many degenerated pyknotic nuclei, which lay in pairs or singly in the epithelium, and they were very difficult to distinguish from dense spireme stages in pro- and telephase. In order somewhat to find out what this course of error may signify, I have recorded in Fig. 6 the number of mitoses

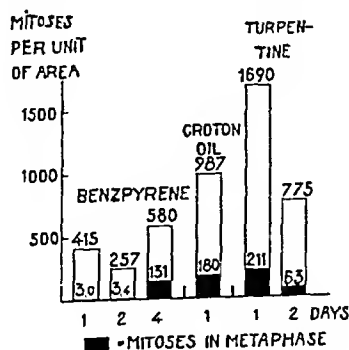


Fig. 6.

Number of mitoses per unit of area.

per unit of area, i. e. per 100 mm. section length of 5 μ thick sections. Here the metaphase stages are recorded as black columns, and the remaining stages by clear columns. From this it is likewise obvious how rare the metaphase stages, i. e. the perfectly sure mitoses are during the first days of Bp treatment, those which do occur often being atypical. After the lapse of from three to four days, however, numerous metaphase mitoses are seen. Their number is of the same range of magnitude as that which is already found during the first 24 hours of turpentine and eroton oil hyperplasia. This difference may explain why the hyperplasia provoked by these irritants appears more rapidly than the Bp hyperplasia.

Thus, if a considerable addition of cells through indirect cell division does not occur in the early stages of Bp hyperplasia, the question arises how this hyperplasia can come about. There are several possibilities, namely, a certain addition probably is brought about by direct cell division — that is substantiated by very suggestive pictures with two or more nuclei in one cell. Further, a great many cells may be displaced to the surface epithelium from the hair follicles just as in wound-healing. However, of the greatest importance must be the pronounced increase of size both of cells and nuclei.

Analysis of the increase of volume of cell nuclei in different forms of epithelial hyperplasia.

A considerable increase of the volume of cells and cell nuclei was emphasized by Orr, Page, Pullinger, and others, as being characteristic of the epithelial hyperplasia after application of carcinogenic hydrocarbons. The determination of the cell volume meets with great difficulties, whereas the size of the nuclei can be measured and calculated with fairly great accuracy. The measuring of the size of the nuclei directly or indirectly even affords a certain idea of the size of the cell.

Fig. 7 shows the mean value for the nuclear size in different layers of cells in different forms of epidermal hyperplasia, the relative figures of size for the cell nuclei being recorded on the Y-axis, and the cell layer from the basal layer and upward on the X-axis. As is seen, all the hyperplasias present a marked increase of the size of the nuclei as compared with normal skin of back. With cantharidin the same maximum quotient is obtained as in Bp hyperplasia, at least in certain cell layers. The occurrence of large nuclei alone thus cannot be specific of Bp hyperplasia.

On the other hand, Paletta, Cowdry and Lischer emphasize that it is not the size of nucleus *per se*, but a greater variation of the size of nucleus within one and the same epithelial layer, which should be characteristic of Bp hyperplasia.

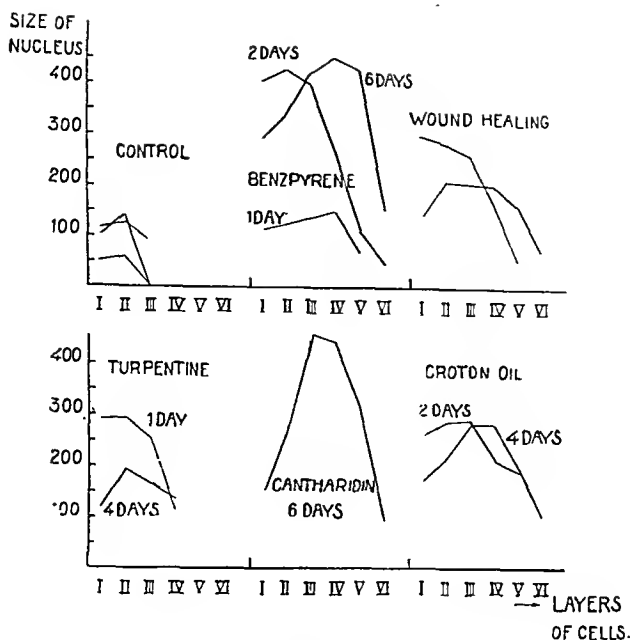


Fig. 7.

Mean value for sizes of nuclei in different layers of cells.

Fig. 8 represents showing the dispersion of the size of nucleus in different forms of hyperplasia. Each histogram comprises the nuclei within a couple of adjacent cell layers. Without too distinct a deviation from the hyperplasia in wound-healing and cantharidin treatment the Bp hyperplasia yet presents the greatest dispersion, which is above all indicated by the size classes 7—10 being amply represented.

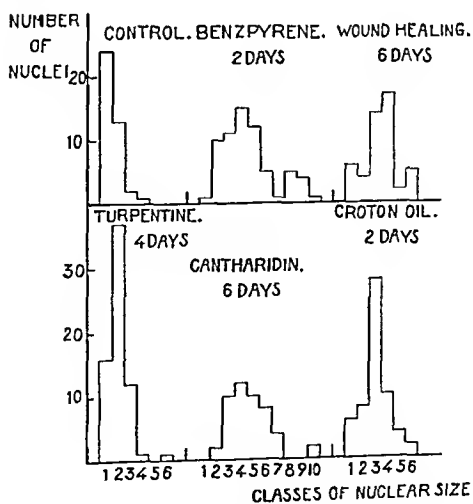


Fig. 8.

Dispersion of the nuclear sizes in stratum spinosum.

Nuclei of this range of size are only met with in a small percentage in other forms of hyperplasia than carcinogenic hyperplasia, Bieseley by examinations of the cell nuclei hyperplasia has found a certain percentage of cell nuclei with doubling of their size associated with doubling of their number of nucleoli and of the number of heterochromatin segments. It is possible that the little group with large nuclei in Bp hyperplasia corresponds to nuclei with double equipment (Fig. 8).

Discussion.

The examination which I have accounted for comprises two phases. One of them was to find out whether the increase of mitochondria was characteristic of the Bp hyperplasia or not.

As regards this question the reaction was found to be the same in unspecific and Bp hyperplasia. We therefore have regarded the reaction as an expression of the increased synthesis of cell constituents which takes place in the rapidly growing cells. The circumstance that this accumulation of lipoids takes place perinuclearly, namely, in the very zone to which the synthesis of cytoplasm protein according to Caspersson and co-workers' investigations is localized, in my opinion seems to suggest that different lipoids may play a part in this synthesis.

It is thinkable that this reaction is connected with the co-carcinogenic action of the majority of the examined skin irritants. The perinuclear increase of lipoids affords the possibility of accumulation of the carcinogenic substances in the very zone which is of so great a metabolic importance and thus creates favorable conditions for the action of the carcinogenic substances. This is a hypothetic possibility, however, and it is just as reasonable to think that Mottram is right in attributing the co-carcinogenic action of croton oil and cantharidin to the great abundance of mitoses which distinguishes the hyperplasias caused by these substances.

Moreover, the co-carcinogenic effect may, perhaps, be explained in another way: it need not at all be related to the epithelial reaction, but it may be due to changes produced in the subcutis (Linell).

The aim of phase II of this investigation was to throw some light on the characteristics which distinguish the Bp hyperplasia from other forms of skin hyperplasia. Thereby, certain features, which are characteristic of the Bp hyperplasia, have become more conspicuous. A summary of my results as compared with those reported by previous investigators affords the following picture of carcinogenic epithelial hyperplasia: The influence of benzpyrene on the skin epithelium is characterized by a strong growth stimulation of the epithelial cells without any more pronounced primary cell injury. During the first,

and particularly during the second day the stimulation manifests itself by a marked increase of size of the single cells, the cell nuclei, and the cell organoids, whereas the indirect cell division is disturbed. Subsequently ensues a lively indirect cell division, whereas the increase of size of the cells and the cell nuclei persists. This increase of size varies in one and the same cell layer, whence the size of the nuclei presents strong disparity. Epithelial hyperplasia caused by unspecific skin irritants does sometimes present a similar picture, but the course of reaction is different, and in my opinion that indicates a certain degree of specificity. It is not the single morphological details, but the course of the reaction on the whole, which seems to be decisive.

Summary.

1) A perinuclear accumulation of lipid-containing cytoplasm structures appearing in the epithelial cells during the development of an epidermal hyperplasia has been studied in the fluorescence microscope. This reaction ensues in the hyperplasia regardless of its being caused by carcinogenic or unspecific irritants (turpentine, croton oil, cantharidin, etc.). An increase and perinuclear condensation of mitochondria are always associated with this reaction.

2) During the development of benzpyrene hyperplasia the epithelial cells show a disturbance of mitoses, probably due to an obstruction at the transition to the metaphase stage. This disturbance is not seen in unspecific hyperplasia.

3) A marked increase of size of the cell nuclei is observed in every hyperplasia but a greater variation of size may be characteristic of carcinogenic hyperplasia.

4) The bearing of perinuclear accumulation of lipid cell constituents on the mechanism of the co-carcinogenic action of unspecific skin irritants and on the protein synthesis of the cells is discussed.

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NO DISCUSSION

ON COLCHICINE TESTS FOR THE PURPOSE OF ASCERTAINING CELL DIVISION AND REGENERATIVE CONDITIONS IN THE LIVER OF THE RAT

By *Harald Teir*.

(Included in a series of papers on the growth and regeneration of cells, partly published in co-operation with Kaarlo Kyllönen, M. B. The remaining articles will also be published in *Acta Pathologica et Microbiologica Scandinavica*).

The tissue of the liver belongs to the organs of the mammal body which, during the entire lifetime, maintain a high power of regeneration. As to the liver of the rat, *Colucci* (1883) removed large parts of the organ, but the removed liver tissue was soon replaced by new, fully functioning tissue, this fact being confirmed by *v. Podwyssozki* some years later. *v. Meister* (1891) removed $7/8$ of the liver of a rat, and from the remaining part regenerated fully functioning tissue to such an extent that the weight of the organ reached normal values.

It is a well-known fact that, as a rule, mitoses are not found in the normal liver of the adult mammal although the organ maintains a high power of regeneration even in old individuals, a fact latest proved by the Americans *Norris*, *Blachard* and *Povolny* (1942) in partial hepatectomy on rats of different age.

In liver tissue injured by various parenchyme poisons such as phosphorus, arsenic and chloroform, as also in parenchymal injuries caused by ligation of branches of the hepatic artery, of some large biliary duct, or of branches of vena portae, mitoses have been found, and regeneration processes in this area have been studied. The most usual object for such investigations was perhaps the latter stages of acute yellow atrophy.

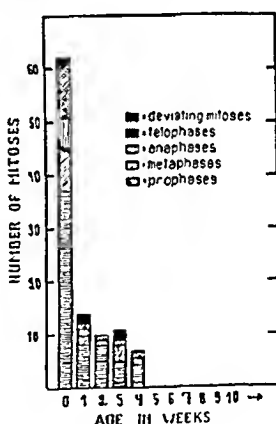
While the epithelium of the intrahepatic biliary ducts was earlier considered mainly responsible for the tissue reproduction of the liver, the opinion that the new liver cells arise out of old, and that the epithelium of the biliary ducts does not change into secretory liver cells, has lately been widely accepted. This opinion of the development of the liver corresponds with that principally represented by *Hammar*

(1926) according to which the liver cells and the intrahepatic biliary ducts develop as different systems.

The karyoclastic poisons have furnished new possibilities for examining the capacity of cell division and regeneration of various organs. For some years I have been making such studies of the liver of the rat.

In the white rat mitoses are not found in the liver of adult individuals. In order to find out when mitoses cease to occur, I investigated first the frequency of mitoses in rats of various age, 52 experimental animals being divided into 15 different age-groups comprising new-born, 1, 2, 3, 4, 5, 6, and 8 weeks old and 3, 4, 5, 6, 7, 8 and 11 months old animals, of which 4—6 rats representing the younger and 2—3 animals the older age-groups.

The experimental animals were all of the same origin. They were similarly fed, and decapitated at the same time of the day. With the aid of binocular 10 and objective 40 I counted, in 150 fields of vision per animal, the number of mitoses in the *Susa*-fixed preparations stained with *Haidenhain's* iron hematoxylin, determining the average number of mitoses of the various age-groups. The result appears from picture 1.



Picture 1.

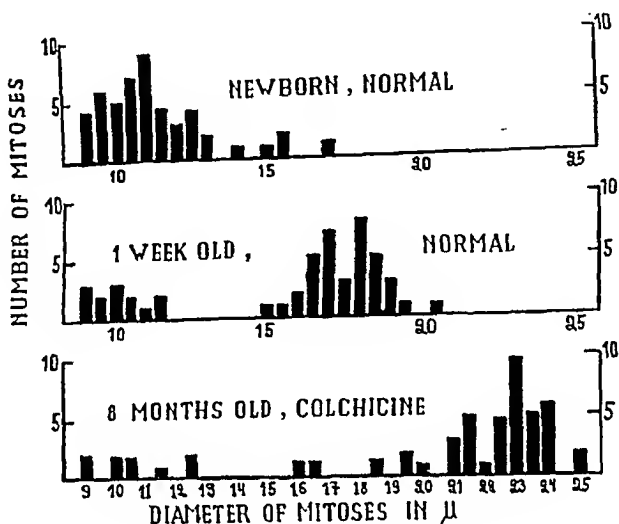
Frequency of mitoses in normal rats.

In the new-born rats the mitotic count was 62, and thus rather large. The differently marked parts of the pillars indicate the number of different mitotic stages, prophase, metaphases, anaphases, telophases and deviating mitoses. At the age of one week the number of mitoses decreased to 14, remaining, during the following weeks, around 10, and beginning at the age of 5 weeks mitoses are, as a rule, not found. However, in one of the oldest rats, 11 months of age, one solitary prophase was observed.

The occurrence of mitoses in the biliary ducts does not, however,

appear from this investigation. Therefore, I examined section series of longitudinally split biliary ducts finding during the first extra-uterine month, about the same number of mitoses as in the liver cells.

I found the three different nuclear classes established by *Jacobj* (1925) in the liver of the rat to be well represented at the age of 4 weeks, thus at an age when mitoses were still observed in the organ. I noticed no certain amitoses.



Picture 2.
Sizes of the mitoses.

I wish to draw special attention to the sizes of the mitoses. At the top of picture 2 can be found the sizes of mitoses in new-born rats. The size of 50 prometaphases, chosen at random, and cut in series, was determined. The diameters were fairly uniform, varying between 9 and 13 μ .

In the one-week rats there are already considerably larger mitotic sizes, most of them ranging about 16–19 μ , as appears from the middle diagram. As I have already proved with regard to the outer orbital gland of the rat (*Teir* 1944), conclusion can be drawn from the frequency maxima in the mitotic sizes that the mitoses of the cells belong to different nuclear classes.

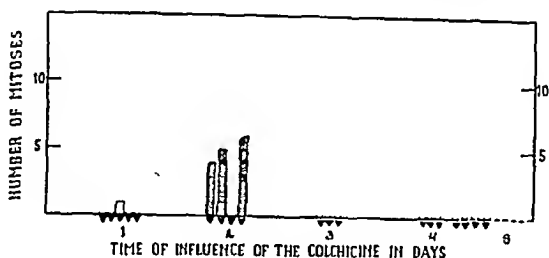
The mitoses in the epithelium of the biliary ducts were small and of equal size. The diameters varied between 8 μ and 12 μ (cf. fig. 6, a).

I shall give an account of three test series of the performed colchicine experiments.

The first comprised 19 white rats of 10 months. The length of the life of a rat is about 2–3 years, these rats thus being middle-aged. As I have previously found the lethal colchicine dosis for my rats to be 0.4–0.8 mg, I injected subcutaneously a sublethal dosis of 0.2 mg, the

animals thus receiving an average of 0.1 mg colchicine per 100 gr body weight. The rats were killed by decapitation in groups of 2—5 animals after 1, 2, 3, 4, 7 and 9 days.

The result appears from picture 3. The abscissa indicates the time of influence of the colchicine in days, and the ordinate the number of mitoses. The black triangles correspond to the distribution of the experimental animals. The white pillars indicate the number of certain colchicine mitoses with a light plasma and stumpy short chromosomes either scattered in the plasma, or collected in larger or smaller clumps.



Picture 3.

Frequency of mitoses after colchicine application.

As appears from the picture, the effect of colchicine is fairly small. Only two distinct colchicine mitoses were observed one and two days respectively after the treatment. After two days a small number of ordinary mitoses with slim prophase chromosomes were further noticed which were not blocked in metaphase (cf. fig. 6, b), as both anaphases and telephases were observed. The reason for the appearance of ordinary mitoses might be the same as in parenchymal injuries caused by phosphorus and arsenic, thus directly regenerative, particularly as I observed in these experimental animals a more or less marked parenchymal degeneration in the liver. The result of this experimental series corresponds, as a whole, with earlier investigations according to which liver cells are fairly colchicine resistant. A further increased mitotic frequency I established when the colchicine was applied in a light ether anesthesia, and biopsy specimen of the liver taken after chloroform anesthesia of a duration of 1½—2 minutes.

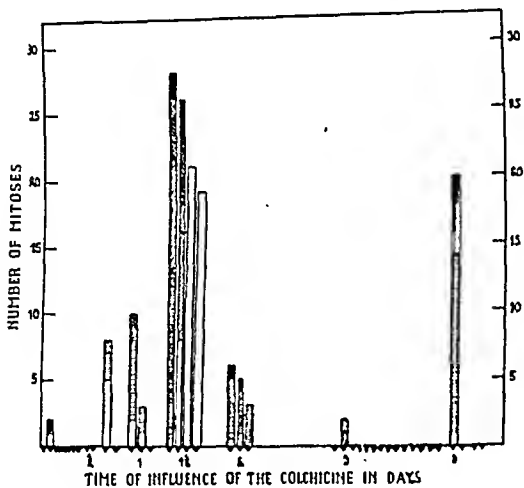
The first test series with colchicine and narcotica comprised 50 white rats of 8 months, of which 23 received 0.2 mg and 27 0.4 mg colchicine subcutaneously. 11 out of the experimental animals which received the larger dosis died during the course of the experiment. The rats were decapitated in groups of 2—4 animals ½, 1½, 2, 3, 4, 5, 6, 7, 8 and 9 days after the colchicine treatment.

The result of this experiment appears from picture 4. There are more colchicine mitoses than in the former test series although they are still fairly sparse. The period between ¾ and 1½ days shows the grēatest number of mitoses. There is, at the same time, often a considerable amount of ordinary mitoses which is not surprising, seeing

that both the narcotica and the colchicine have a toxic influence on the liver tissue.

These colchicine mitoses make certain observations on the mitotic division of the liver cells possible.

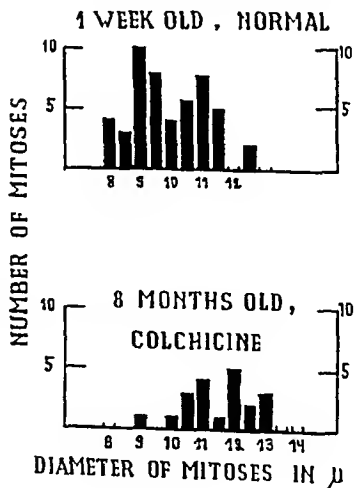
The mitoses are often of a striking size (cf. fig. 6 c and d). Fig. 2 shows how a majority of the mitotic diameters in 8 months old col-



Picture 4.

Frequency of mitoses after colchicine application with narcotica.

chicine treated rats form a frequency maximum at 21—24 μ . In serial sections of a thickness of 5 μ of such mitose I counted as many as 90 chromosomes. As the diploid number of chromosomes of the rat is 42, it is probably here a question of octoploid cells and probably of cells of the third nuclear class. It remains accordingly a considerable, de-

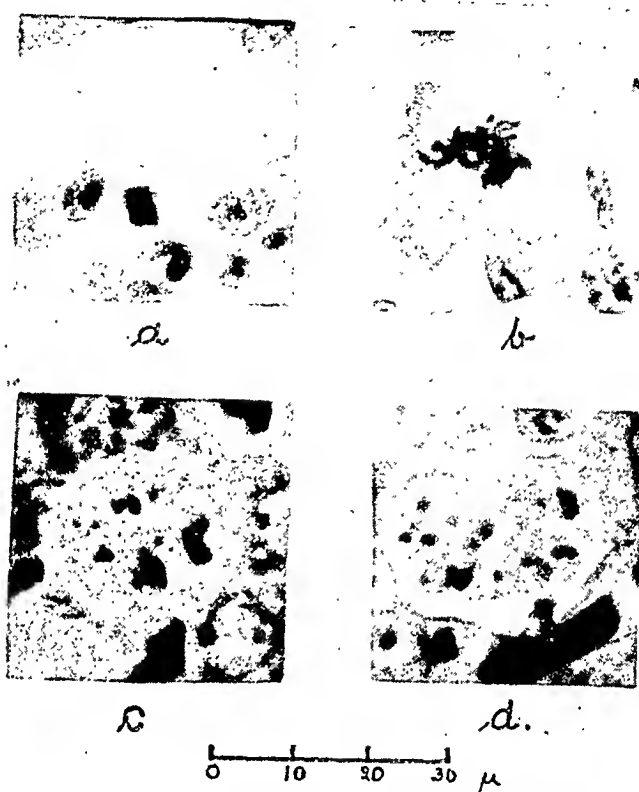


Picture 5.

Sizes of mitoses in the epithelium of the biliary ducts.

ficit of the expected theoretical number of the chromosomes 168. A small number of cells were of a size equal to those which we found predominating in new-born animals.

In the epithelium of the biliary ducts I found surprisingly few colchicine mitoses and mitoses in general. Those I saw were all small.



Picture 6.

a = a mitosis in the normal epithelium of a biliary duct in a 2 weeks old rat; b = a prophase with slim chromosomes in a 10 months old colchicine rat; c and d = typical large colchicine mitoses in 8 months old rats. — All pictures enlarged 1000 \times . *Susa-Heidenhain's* iron hematoxylin.

Picture 5 shows at the top the mitotic sizes in the epithelium of the biliary ducts in normal 1 week old animals. There is a frequency maximum at 10–13 μ .

The lower picture illustrates the mitotic sizes in the epithelium of the biliary ducts in 8 months old colchicine treated rats, and the sizes correspond well with those of the normal mitoses.

Mitoses are fairly often found in the periportal and perivascular connective tissues of colchicine animals. *Cavallero* (1939–40) who, with the aid of colchicine, examined cirrheses produced by carbon

tetrachloride, found the *Kupffer* cells to be the origin of the connective tissue proliferation. In my experiments these cells appeared to be fairly colchicine resistant.

In the third experimental series 12 2 months old rats received 0,2 mg colchicine subcutaneously in aether anesthesia, this dosis corresponding, on an average, to 0,15 mg of the poison pro 100 gr body weight. At the age of 2 months the rat is sexually mature even though the physical development is still not quite finished. The biopsy specimens were taken in chloroform anesthesia 5,8, 12, 18 hours and 2, 4, 5 and 7 days after the treatment. Already after 5 hours a strong reaction was observed, the chromatine appearing distinctly concentrated, and the nuclei being uneven with ragged contours. A small number of typical colchicine mitoses were observed after 18 hours. After an interval of 18 hours almost every nucleus seemed pyenotic. After one week there were still signs of parenchymal degeneration, the number of the ordinary mitoses being determined at 7.

From these experiments it appears that the liver cells of the white rat do not loose their power of mitotic division even though, to some extent, it decreases with increasing age. Particularly the large cells are prepared for mitosis, a circumstance which well corresponds with a fact frequently stressed, viz. that remarkably large cells are found in liver tissue.

The epithelium of the intrahepatic biliary ducts does evidently not possess an equally high power of mitotic division as the liver cells. By determining the mitotic sizes one may obtain an approximate idea of the origin of the new-formed liver tissue.

It is evident that the liver cells on the one hand and the epithelium of the biliary ducts on the other, regenerate separately without fusing.

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DISCUSSION

R. FÄHRÆUS: The interesting paper of Teir, I think, suggests the question as to whether it be justifiable from an increased number of mitotic figures to conclude that the mitotic intensity of the cells has increased. Indeed, one might just as well imagine that it signifies a slowing-down of the mitoses.

J. MELLGREN: With a view to the question about the mode of action of colchicine I wish briefly to give an account of an experiment which I performed in this institute in 1943, on the *relation between the rate of the growth of the adrenal cortex and the frequency of mitosis under treatment with colchicine*.

The males of a uniform strain of white mice were divided after their age into 7 groups, each comprising at least 15 (usually 30) animals with no age difference exceeding 14 days. From each group, the 5 animals were picked out which in body weight came nearest the medium weight for the group. All the selected animals were given 0.002 mg. colchicine per. g. body weight, subcutaneously, and decapitated 9 hours later. The adrenals were fixed in 20 % formalin for 3 hours, embedded in paraffin, cut in sections of 5 μ , and stained with Harris' hematoxylin. Every fifth section was projected on a screen at a certain magnification, and the volume for the cortex was calculated after Hammar. In one section, near the center of the cortex, the density of cells was counted as well as the number of mitotic figures. The absolute number of cortical cells per adrenal was calculated from the values for volume and density of cells (with corrections for thickness of sections, etc., according to Carlson *et. al.*, Upsala Läkarefören. förh. Ny följd 43, 49, 1937).

The result is evident from Fig. 1, in which the upper full-line curve signifies the number of cortical cells (= the medium of 2

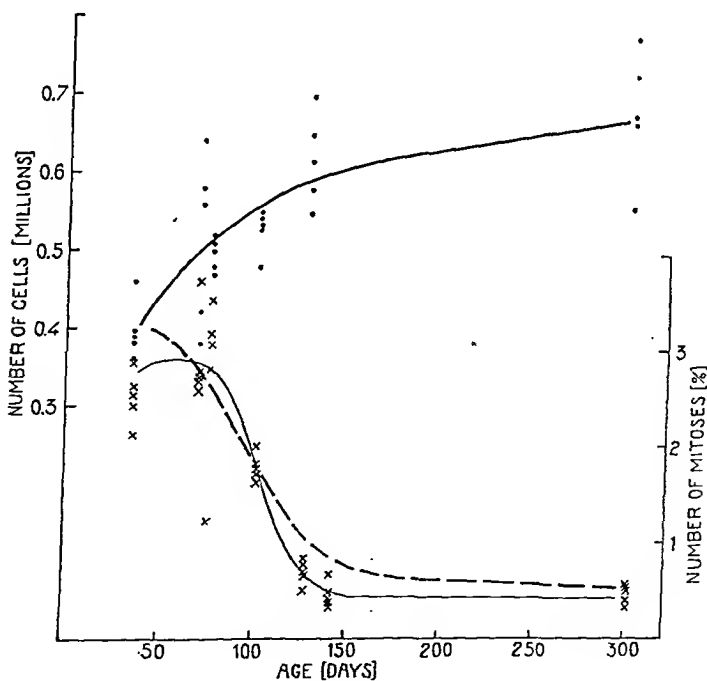


Fig. 1.

adrenals = 1 animal). The lower broken curve (-----) is derived from the upper, *i. e.*, calculated rate of addition as function of the age. The lower full-line curve (——) signifies the empirical frequency of mitosis as a function of the age (x = medium of 2 adrenals = 1 animal). As will be noticed, with the scale here adopted the two lower curves turn out fairly identical.

So in male mice under colchicine treatment the frequency of mitosis in the adrenal cortex is largely proportional to the rate of growth. From this it certainly is not evident whether colchicine promotes the appearance of more mitoses than normally, or merely prolongs the course of each mitosis. But, on the other hand, the experiment shows that the frequency of mitoses under treatment with colchicine really may serve as a measurement for the rate of growth — something that was assumed previously without really being corroborated experimentally.

H. TEIR: The mitotic time of the somatic cells of the mammals is estimated at about 30 minutes. The mitotic division of large cells probably requires more time. The mitotic time of the colchicine mitoses which are blocked in metaphase, is considerably longer, a fact which is apt to increase the number of mitoses. As appears from fig. 1, however, mitoses are not found in the liver of adult rats. Therefore, the presence of mitoses after colchicine treatment of 8 to 10 months old animals shows that the colchicine both here and — as I found also to be the case — in the outer orbital gland of the rat, *stimulates* the cell to mitotic division even though the cell is later blocked in metaphase. In such a case as this the cells which have maintained their power of mitotic division, naturally react. Of course the number of colchicine mitoses of different cells in a tissue cannot be directly compared with the number of mitoses in normal tissue, but it indicates primarily the degree of the mitotic preparedness of the cells in question, and is consequently also a gauge of the regeneration power of these cells.

THE RELATION OF THE SYNTHESIS OF HEMOGLOBIN TO THE CELLULAR GROWTH DURING NORMAL AND CERTAIN PATHOLOGICAL CONDITIONS

By B. Thorell.

To maintain a constant number of red blood cells in the blood stream a considerable production in the blood-forming tissue is necessary; in the adult man c. 100 mill. erythrocytes are formed per minute. As 33 % of the erythrocytes consist of hemoglobin, the continuous formation of hemoglobin can be calculated to be several milligrams per minute.

In principle the formation of hemoglobin-containing blood cells takes place from a relatively small and constant number of stem cells, which produce a large number of mature blood cells by means of division and maturation through morphologically characteristic stages. The formation of mature blood cells from the stem cells in the bone marrow involves the two fundamental processes of cell renewal, partly growth by increase in size and division and partly differentiation into a functionally specialized tissue cell.

In the present investigation the question of the relation of the growth processes to the specific cell-differentiation processes is attacked with the help of quantitative ultramicrochemical methods of analysis. With these methods it is possible to investigate the processes within the single cell which are associated with 1) cellular *growth*, defined as the new formation of the fundamental constituents of the cell, and 2) *differentiation*, defined as the formation of the cellular substances which make it possible for the cell to perform its specific function.

Ad 1. The synthesis of the fundamental constituents of the cell, as for example the cytoplasmic proteins, during intensive growth occurs in the presence of high concentrations of ribose nucleic acids. (Caspersson and co-workers 1939—1945)*). The nucleic acids have a high selective light-absorption in the ultraviolet spectral range at 2600 Å. This makes it possible to estimate directly the quantity and distribution of nucleic acids in a living cell by micro-spectrophotometry.

*) For references, see Thorell, Acta Med. Scand., Suppl. CC, 1947.

metrical analysis. The results of such analyses have shown that the growing cell develops a special organization for its nucleic acid metabolism, in which the nucleolus, the nuclear membrane and the cytoplasmic ribose nucleic acids are essential parts. Thus, an intensely growing, protein-forming cell is characterized by a large ribose nucleic acid-containing nucleolar mass, a large nucleus with a well-developed nuclear membrane and high concentrations of ribose nucleic acids in the cytoplasm.

This is the situation also during erythropoiesis. In the immature stem-cell high concentrations ($> 5\%$) of cytoplasmic ribose nucleic acids can be demonstrated. The nucleolar apparatus contains considerable amounts of ribose nucleic acid.

During later phases of development, however, the concentration of cytoplasmic nucleic acids decreases rapidly; already in the stage

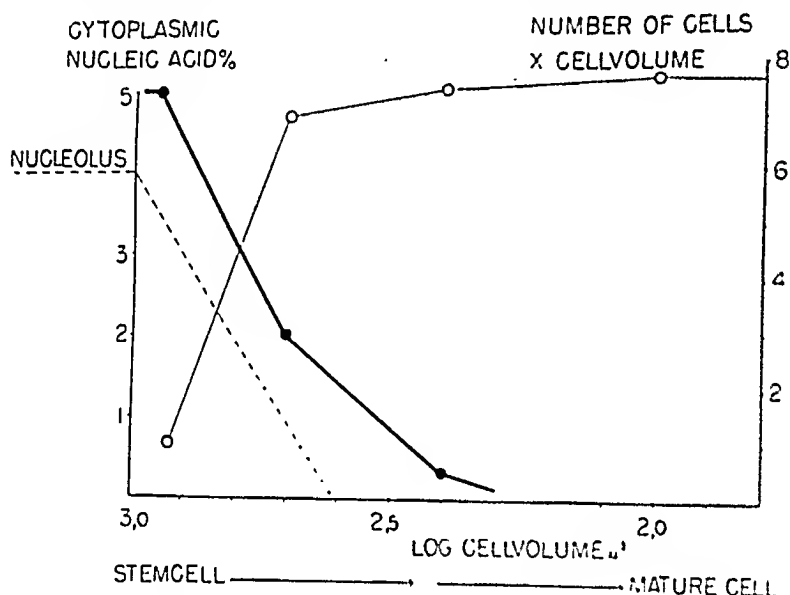


Fig. 1.

Survey of the essential cytochemical and cytological changes connected with the growth processes during erythropoiesis. Abscissa: logarithm of the volume of the single cell. According to the measurements the log cellular volume of the proerythroblast is c. 2.95, basophilic erythroblast 2.70, polychromatic erythroblast 2.40 and orthochromatic erythroblast 2.0. The division into log cellular volume is chosen, as it under certain conditions is proportional to time.

The curve —o—o— shows that the vastly predominating increase in cellular substance during erythropoiesis occurs during the maturation of the proerythroblast into the basophilic erythroblast. The increase is c. 8 times. From the diagram it appears that before and during this principal increase in cellular mass the cells are characterized by a large nucleolar mass and high concentrations of ribose nucleic acid in the cytoplasm. During the course of maturation the nucleolar mass and the cytoplasmic nucleic acids decrease parallel with a cessation of the growth activity of the cell.

which morphologically is called »polychromatic« the nucleic acid concentration can no longer be measured. The ribose nucleic acid-containing organelles of the nucleus have also disappeared.

These cytochemical data, along with the quantitative cytology of the bone marrow, can be correlated with the intensity of formation of cellular substances during erythropoiesis. The high concentrations of cytoplasmic nucleic acids in the earlier developmental stages correspond to the relatively greatest increase in cellular mass (see fig. 1).

From a quantitative point of view, both the micro-spectrophotometrical and the cytological analyses permit of the conclusion that the intensity of growth during erythropoiesis is proportional to the amount of nucleolar substance and to the concentration of cytoplasmic ribose nucleic acid. From this, there follows an alternative possibility of determining the growth conditions of an individual erythropoietical cell by analysis of the distribution and concentration of the ribose nucleic acids in the cell.

Ad 2. From the functional point of view the processes of differentiation during the formation of the red blood cells are characterized principally by the formation of hemoglobin. If the hemoglobin content within each individual cell is measured during the different developmental phases in erythropoiesis, a measure is obtained of the degree of differentiation of the respective types of cells, expressed in a quantitative cytochemical unit.

The light absorption of hemoglobin affords good possibilities for micro-spectrophotometrical estimation of the hemoglobin content in a single cell. At the Soret-band (4100 Å) the decadic extinction coefficient of hemoglobin is about 10^4 . This gives, in a layer corresponding to the thickness of a cell and in the range of concentration from 0,5 % to 33 % hemoglobin, a measurable diminution in the intensity of light ($5 \% < I < 95 \%$).

The principle of the cytochemical hemoglobin analysis lies in the photoelectrical measurement of the intensity of light in a microscope-optically enlarged image of the cell which is projected on a photocell aperture. The diameter of the photocell aperture corresponds to an area in the cell of $0.2 \mu^2$ (fig. 2). By this arrangement the distribution of light-absorbing substances within the cellular structures can be determined with fairly great accuracy. Owing to the fact that the living cell is almost optically empty in the visible spectral range, the light absorption of the cell at the Soret-band is dominated by the absorption of the hemoglobin. This makes it possible to calculate the total amount of hemoglobin from the »total light absorption« which is obtained by integrating the light absorption values over the cellular volume.*)

*) For details, see ref. above.

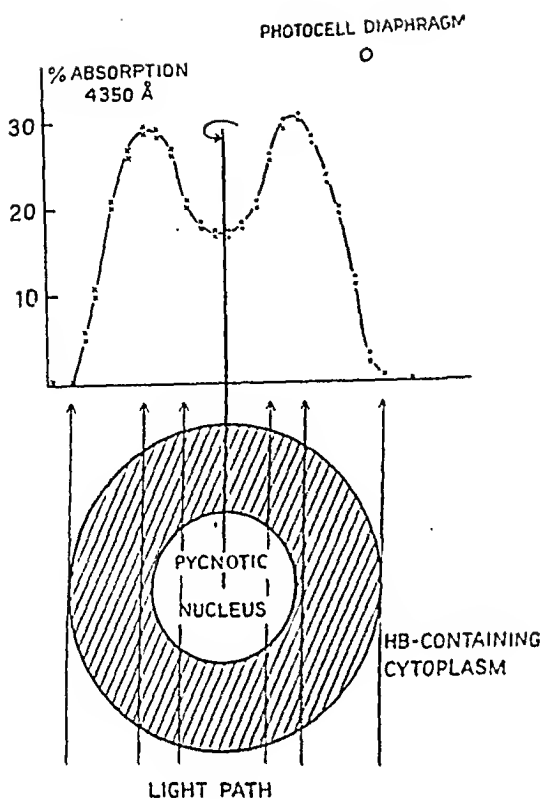


Fig. 2.

The principle for hemoglobin determination in a living erythropoietic cell. Points along the diameter of the image of the cell are thrown on a photocell aperture. The light absorption is calculated from the photocurrent. Knowing the dimensions of the cell, the hemoglobin concentration in each point along the cell-diameter can be calculated from the light absorption values. By integrating the measurement curve over the cellular volume, as indicated by the arrow, the total amount of hemoglobin in the cell can be calculated.

Fig. 3 shows the hemoglobin content in the different developmental phases together with the ribose nucleic acid values. The changes in the cytoplasmic composition during erythropoiesis show firstly, that before the processes which lead to the cellular differentiation in the direction of hemoglobin formation have begun, the metabolism associated with the new-formation of the cellular mass is completed. Secondly the diagram shows that the increase in total amount of hemoglobin per cell unit does not take place uniformly.

Thus on quantitative cytochemical basis the unipotent red cell development occurs in several different phases as regards the intensity of cellular growth and differentiation.

During the first phase, the growth phase, it is principally the formation of the basic cell protein substances that takes place. The cell then has a cytochemical organization resembling that of growing

cells in general (see above 1). The next phase involves a decline in the growth processes, cytochemically observable as a rapid decrease in the concentration of the cytoplasmic ribose polynucleotides and the nucleolar mass. At the end of the decline in growth, the first signs of the processes which lead up to the differentiation of the cellular

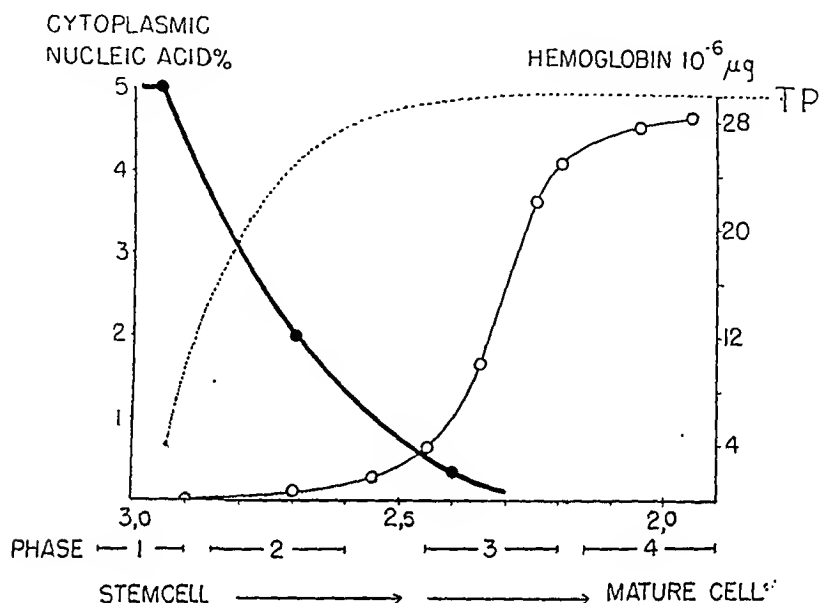


Fig. 3.

Survey of the essential changes in cytoplasmic composition during erythropoiesis. Abscissa as in fig. 1. —●—●— cytoplasmic ribose nucleic acid %, —○—○— total amount of hemoglobin in the cell. TP total cellular protein calculated from the total volume of cells in each development phase (cf. fig. 1). The figure shows on the whole, that before the erythroid cell is differentiated into its final functional stage, the ribose nucleic acid metabolism associated with the endocellular growth processes is finished, *i. e.* that during the uni-potent cell development the new formation of the cellular protein substances is completed *before* differentiation occurs into its final, specific form. For details, see text.

proteins into their final specific forms begin to be observable. These processes become increasingly rapid, and at the phase where the concentration of cytoplasmic nucleotides reaches the zero value, a great generation of hemoglobin sets in — the differentiation phase. When the cell has reached a degree of differentiation corresponding to c. $23 \cdot 10^{-6} \mu g$ of Hb, the formation of hemoglobin declines —, the phase of declining differentiation. The total amount of hemoglobin in the cell approaches a value of $28 \cdot 10^{-6} \mu g$, corresponding to that of the finished erythrocyte. Thus, in the last stage with pycnotic nuclear degeneration and during the denucleation process to the erythrocyte of the blood, the hemoglobin content of the cell does not increase.

The concentration becomes greater (from 25 to 33 %), owing to the decrease in volume during this process.

Thus it is possible to follow quantitatively the processes which form the basis of the normal growth and differentiation of the blood cell. The next question is whether any changes in these processes can be detected in cases of pathological disturbances during erythropoiesis.

The types of pathological erythropoiesis investigated were pernicious and chronic hemorrhagic anemias. Four clinically unequivocal, untreated cases of pernicious anemia were examined. For the investigation of hemorrhagic anemia adult rabbits were used from which about 50 cm³ blood was tapped every other day for three weeks.

The living bone marrow cells were analyzed as described above, with micro-spectrophotometry in ultraviolet for determining the ribose nucleic acid concentration in the cytoplasm and with micro-spectrophotometry in the visible spectral range for determining the concentration and amount of hemoglobin. The different types of cells were classified, as above, according to their volume.

The changes in the processes of endocellular growth and differentiation, as shown by the results of the analyses of pernicious and hemorrhagic anemia respectively, are most easily displayed by inserting the values obtained in a system of coordinates similar to that which was used for the interpretation of the cytochemical analysis data of normal erythropoiesis. The diagrams in fig. 4 were obtained in that way.

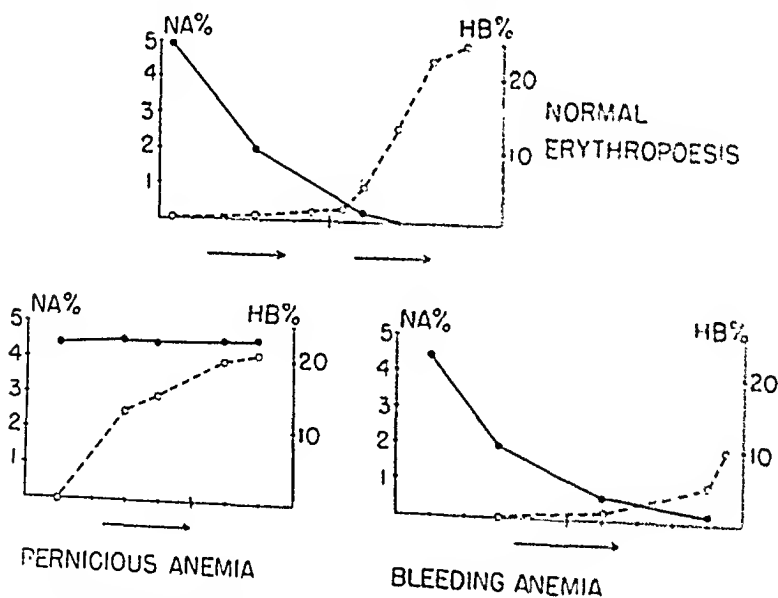


Fig. 4.

Survey of the changes in the processes of endocellular growth and differentiation in the case of pernicious and hemorrhagic anemia. Abscissa as in fig. 1 and 3. NA % = concentration of cytoplasmic ribose nucleic acid, HB% = concentration of hemoglobin in the cytoplasm.

During normal erythropoiesis there is a definite relationship between the growth and differentiation processes of the cellular substances. All the cases of pernicious anemia investigated show a clear and identical disturbance in this relationship (see fig. 4).

The process of formation of cellular substances in these cases is clearly characterized by the non-disappearance of the ribose nucleic acids of the cytoplasm and nucleolar apparatus. Normally the ribose nucleic acids disappear during erythropoiesis and this begins during an early phase of maturation. On the other hand, formation of hemoglobin seems to take place in the bone marrow cells in pernicious anemia. Thus in this disease there are cells enriched in the blood-forming organ with comparatively high concentrations of cytoplasmic nucleic acids; in some of these cells, the synthesis of hemoglobin has proceeded relatively far.

The disturbances in the interplay between the growth and differentiation processes of erythropoiesis in the case of hemorrhagic anemia are of a different type. The decrease in the nucleic acid metabolism of cell growth proceeds normally, while on the other hand the hemoglobin synthesis in the cell exhibits a low intensity.

Thus the analyses show the possibility of attacking, with quantitative microspectrographical methods of analysis, certain disturbances in the relationship of the endocellular differentiation processes to the growth processes. The analyses also show that these disturbances may be looked upon as being specific for different types of pathological conditions during erythropoiesis, manifesting themselves as pernicious and hemorrhagic anemias respectively.

DISCUSSION

Å. WILTON: As shown by Caspersson and collaborators, the prerequisite of new-formation of gene-carrying chromosome protein, i. e., formation of mitosis, is the presence of desoxyribose-polynucleotides, whereas ribose-polynucleotides are required for the formation of cytoplasmic protein. Through spectral-analytical studies Dr. Thorell has previously shown that the ribose-polynucleotides in the cytoplasm of the red bone-marrow cells under the progressive differentiation of the cell successively decrease in amount and finally disappear completely. To-day he has presented some studies on the ribose-polynucleotide content as compared to the hemoglobin content of the red cells, likewise determined by spectral analysis. As we just heard, these investigations gave the result that under physiological conditions the ribose-polynucleotides decrease in the same degree as the hemoglobin content increases, and consequently the hemoglobin content increases with advancing cell differentiation. Through these cytochemical investigations Dr. Thorell has arrived at the same conclusion as I formed

through morphological studies. My conclusion was based on investigation into the development of the red cells in human fetuses, chicken and swine, and the preliminary account of these studies was presented at the Scandinavian Congress of Pathology in Copenhagen 1938. It will be appropriate here, I think, briefly to touch upon this subject.

In smears of bone marrow it is not practicable to distinguish between immature cell forms belonging to the red series and to the leukocyte series, as the immature cells of both series are of the type which in clinical hematology is designated as myeloblasts. In birds, however, red bone-marrow cells develop within the vascular wall, leukocytes outside the wall. So when in a paraffin section of chicken bone-marrow we meet with myeloblast-like cells located inside the vascular wall, we know that they are immature cells belonging to the red series. Such cells have a large vesicular nucleus with one or more large nucleoli and only a little cytoplasm, which is strongly basophil and contains no hemoglobin. In other words, the hemoglobin content of the cell is nil. The progressing cell differentiation can be followed through the gradual alteration of the nucleus from the type just mentioned to the small oval and compact nucleus. One of the intermediate stages is the spoke-formed nucleus, typical of the red cells. When the formation of hemoglobin has commenced, it is possible by means of the shades of the cytoplasm stained after Giemsa to estimate approximately the hemoglobin content of the cells. With a slight hemoglobin content the cytoplasm shows a faint bluish-lilac, which through lilac and orange goes on to the homogeneous red colour of the mature cells. These shades appear particularly distinct in smears from chicken bone-marrow.

My material comprised 54 individuals of fowls, 17 of which were 1-day-old chicks, while 17 were full-grown fowls. The remaining animals belonged to the intermediate age classes. In these studies I found very good agreement between, on one side, the nuclear changes appearing in the process of differentiation and, on the other side, the changes in the color-shades of the cytoplasm. On the basis of these observations I drew the conclusion that under physiological conditions the hemoglobin concentration increases with advancing cell differentiation, so that in normal cases the hemoglobin concentration may serve as a measurement for the degree of differentiation. As we just have heard, Dr. Thorell has arrived at the same conclusion on going an entirely different way — that is — through sensitive spectral-analytical methods.

As the cells circulating in the blood possess the highest hemoglobin concentration, from these investigations I assumed that the saturation index — *i. e.*, the mean value for the hemoglobin concentration of the individual red blood cell — might be looked upon as a measurement for the degree of differentiation of the hemoglobin-forming tissue. In order to investigate the changes in the degree of differentiation

appearing during the ontogenetic development, I employed, among other animals, also swine for my experiments. Through continuous examinations on the same sucking pigs — for this and other purposes — the hemoglobin values, red blood counts, colour index and size of the cells were established in a scientifically reliable manner. This material consisted of 18 litters of sucking pigs, comprising 120 animals. Furthermore, the corresponding blood values were determined on 15 adult swine.

Table 1.

Age	Haemoglobin value	Red blood counts	Colour index	Size of the red cells
Pigs of 8—9 weeks	$72,1 \pm 0,9$ (131) *	$6,71 \pm 0,08$ (80)	$0,53 \pm 0,01$ (80)	$6,4 \pm 0,05$ (35)
Adult swine	$88 \pm 2,0$ (15)	$5,43 \pm 0,19$ (15)	$0,83 \pm 0,03$ (15)	$6,0 \pm 0,10$ (11)

*) The figures in brackets indicate the number of determinations.

As will be noticed from table 1 the color index, *i. e.* the mean value for the hemoglobin content (by weight) of the red blood cells is smaller in the pigs than in the adult swine, whereas the converse applies to the size of the cells. When now the red blood cells of adult swine are smaller than those of young pigs, and yet they contain larger amount of hemoglobin by weight, the hemoglobin concentration in the red cells of the adult swine must be higher than in the pigs. On account of these and other observations my conclusion was: the degree of differentiation of the hemoglobin-forming tissue increases during the ontogenetic development.

With his spectral-analytical methods Dr. Thorell has studied the development of the red blood cells in hemorrhagic anemia and in pernicious anemia. In this way he has made the interesting observation that in pernicious anemia the ribose-polynucleotides in the cytoplasm of the red cells do not decrease in the same way as under physiological conditions. As illustrated very well by the last picture shown by Dr. Thorell, in pernicious anemia there is an abundant of ribose-polynucleotides in the cytoplasm even with a high hemoglobin concentration — in contrast to the findings in hemorrhagic anemia. This observation may imply that here we have available a sensitive spectral-analytical method for the diagnosis of pernicious anemia and its degree merely by examining the proportion between the ribose-polynucleotides and the hemoglobin of a single cell. To me, however, this observation is more interesting from a tissue-biologic point of view. How does it happen, then, that in pernicious anemia the cytoplasm contains plenty of ribose-polynucleotides in spite of the high hemo-

globin concentration. For a satisfactory explanation of this fact, I think, it will not be sufficient merely to study the ribose-polynucleotide content, without studying also the desoxyribose-polynucleotide metabolism. I base this view upon some morpho-biological observations of my own that briefly will be reported as follows.

At an early ontogenetic stage, under physiological conditions, only immature red bone-marrow cells undergo mitosis. In connection with advancing ontogenetic development, however, also the more mature cells, rich in hemoglobin, divide, *i. e.*, the mitosis formation shifts into the direction of the differentiated side. This biological principle, which I have designated as shifting of the mitosis formation to the right, may be illustrated most simply by means of the schematic drawing in Fig. 1. Here stage I represents an early ontogenetic stage, stage II a subsequent stage, and stage III an adult stage. In stage I only the immature red cells divide, and their daughter-cells enter the blood stream. In stage II also the daughter-cells undergo mitosis, and in stage III even the red cells rich in hemoglobin. As suggested by Fig. 1, owing to the shifting to the right of the mitosis formation, during the progressing ontogenetic development the red cells become smaller and smaller.

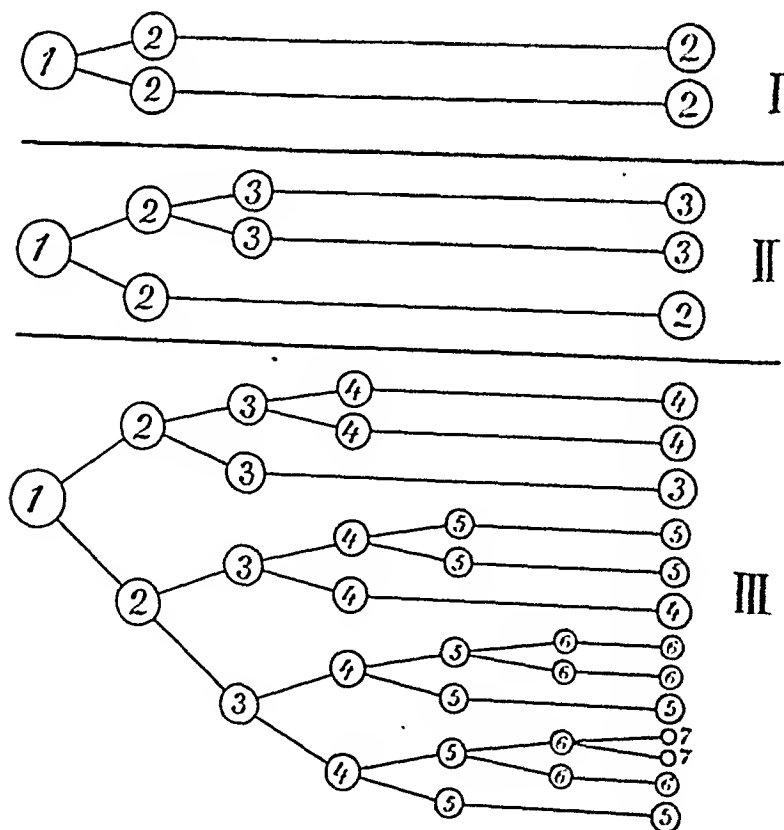


Fig. 1.

Examinations on pernicious bone-marrow before and after administration of antipernicious factor showed that lack of this factor makes the more hemoglobin-rich cells lose their capacity for division under physiological conditions. This observation may be illustrated by means of the same picture (Fig. 1). As previously, stage III shows physiological blood cell development in the adult stage. Stage II shows the blood cell formation in pernicious anemia and also stage I in a severe case of longer duration. As is evident from this picture, in pernicious anemia there is a shift in the mitosis formation to the undifferentiated side («shift to the left of mitosis formation»), which shift offers an explanation as to why the red blood cells increase in

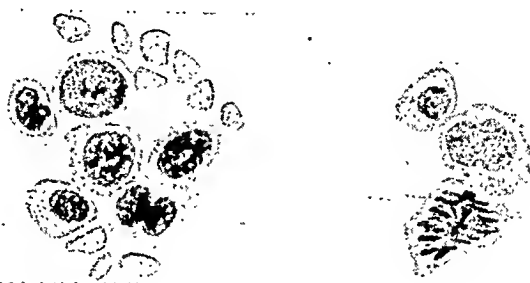


Fig. 2.

Red cells with atypical mitosis in pernicious anemia.

size with increasing severity of the pernicious anemia. But this does not mean that the blood cell formation has returned to the embryonal type, for the hemoglobin concentration in the red blood cells (saturation index) is the same in pernicious anemia as in normal adults, *i. e.*, higher than at the embryonal stage.

But, now, how are we to explain this shifting of the mitosis formation to the left?

Starting from Caspersson and collaborators' observation, that the presence of desoxyribose-polynucleotides is prerequisite to new formation of chromosom protein — *i. e.*, mitosis formation — the shifting to the left of the mitosis formation in pernicious anemia may be interpreted to this effect: that the amount of these nucleic acids in the more hemoglobin-rich cells is not sufficient for physiological mitosis formation. This view finds support in the fact, well known to any hematologist, that in pernicious anemia the hemoglobin-rich cells present a phenomenon, the so-called amitosis or atypical mitosis, which fails to go on to cell division (Fig 2). On administration of anti-pernicious factor, the mitosis formation again becomes physiological.

Great interest is attached to the view advanced by the cytologist and physiologist la Cour: that pernicious anemia is induced by nucleic acid starvation — as he calls it. As far as I can see, with this expression he means a lowered content of the desoxyribose-polynucleotides.

Unlike Dr. Thorell, la Cour has had no opportunity to employ Caspers-sons spectral-analytical methods. By means of certain staining methods, however, he has thoroughly studied the mitosis formation in the bone-marrow in pernicious anemia, and thus he had found certain points of resemblance between, on one side, the mitosis formation just mentioned and, on the other side, mitosis formation in plants, in which »nucleic acid starvation« was produced experimentally. As mentioned, his conclusions are based on staining methods, and thus they cannot be looked upon as being just as reliable as if spectral-analytical methods had been employed. So it would be highly interesting if Dr. Thorell with his great experience in the employment of these methods would extend his investigation to cover not only the ribose-polynucleotide content of the red cells, but also their desoxyribose-polynucleotide metabolism under physiological conditions and in pernicious anemia.

I. WALLGREN: The curves presented by Dr. Thorell, showing that in untreated cases of pernicious anemia the protein production in the erythroblasts proceeds longer than normally, appears quite to agree with my observation. In severe untreated cases of pernicious anemia I have seen new erythrocytes arise through budding from the cytoplasm of megaloblasts and normoblasts. In such cells perhaps also the protein formation was increased.

As to Dr. Thorell's remark about an increase in the uric acid concentration of the blood when the erythropoiesis is increased, I beg to be allowed to deal with this question to-morrow, when presenting my own paper.

TUMOURS OF TACTILE END-ORGANS

By *Erkki Saxén.*

The references to the occurrence of tactile corpuscles in tumours are very scarce in medical literature if we except those dealing with corpuscular structures found in naevi, neurinomas and glomic tumours.

It was already in 1899 that *Soldan* paid attention to the fact that neurofibromas almost regularly appear in association with pigmented naevi. He actually regarded the pigmented naevi as tumours occurring in the connective tissue sheath of the terminal ramifications of the nerve, i. e. as neurofibromas. This view was given no appreciable degree of attention until *Masson* (1926) proved that the pigmented naevi are neurogenic. In his opinion they were not produced as a result of the fibromatous proliferation of the connective tissue; he regarded them rather as ectodermal tumours. He observed that corpuscular structures, which he called »lames foliacées«, were regularly present in them. According to *Masson* these corpuscles resembled distorted *Wagner-Meissner* corpuscles in which the modified *Schwann* cells of the organ play the dominant part.

Also palisaded nodules seen in the neurinomas have been regarded as organoid productions comparable with the tactile corpuscles.

Mention shall be made here also of the glomic tumours in spite of the fact that their structure is only imperfectly known. Nor is it known with any certainty whether they are primarily of nervous or of vascular origin. According to *Bailey* there exists a close analogy between glomic tumours and pigmented naevi. According to *Foot* (1940) glomic tumours and melanomas are tumours of nerve terminals.

In addition to these three types of tumour I have found in the literature accessible to me references to eight separate tumours with formations similar to tactile end-organs. I shall give a brief review of them.

Flörcken & Steinbiss (1921). A man 30 years of age. At the age of 15 a tumour had developed in the middle of the scalp. He was operated on and the tumour was found to be 22 cm. in diameter and 8 cm. thick, »just like

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a cap on the patient's head. It was diagnosed as elephantiasis neurofibroma. The corpuscles resembled in some degree the tactile corpuscles found in the duck's bill. Thin septa which stained blue in Mallory's solution projected in a fan-like way from a sheath composed of fine fibrils. At the edge of each septum there was a comparatively large chromatindeficient nucleus, clearly distinguishable from the nuclei of the surrounding connective tissue and quite similar to those of the Schwann-sheath. The matter between the lamellae was quite homogenic and stained slightly blue. In some corpuscles a small inner body (Innenkörper) was seen. Flörcken & Steinbiss hold the view that the corpuscles are neurogenic, resulting from the productive growth of the Schwann-cells of the peripheral nerves.

Masson (1931). A child of 3, who had died of broncho-pneumonia. Autopsy revealed on the whole scalp a limited, soft, and flat thickening, which fluctuated slightly. The dermis and hypodermis were strongly thickened, forming a fibrous mass, about 2 cm. thick. The tumour contained a great number of naevus cells, tactile corpuscles and plenty of pigmentation. The corpuscles were never innervated and their structure resembled the supporting structure of Wagner-Meissner end-organs. Masson regarded the tumour as a pigmented naevus or rather as a neuromaevus which differed from a common naevus in that it had a deeper lying plexiform neurinoma formation and large tactile corpuscles.

Jordan (1932). A man 18 years of age with a congenital extensive pigmented area in the region of the shoulders. The skin was nodular and thickened in the pigmented area. The scalp was covered with pigmented nodules. The tumours were diagnosed as »Masson's« neuromaevi. Deeper in the loose subcutaneous layers, which stained yellowish in v. Gieson's solution and also free in the connective tissue a large number of peculiar structures were encountered. They were in the majority of cases solitary, appearing only seldom in conglomerates or strings. The inner part of the corpuscles stained light yellow, it had lamellar layers with thin septa running in them. The septa were sometimes provided with nuclei. The nuclei were situated as a rule at the periphery and sometimes congregated only on one side of the corpuscle. In the nerve sheath consisting of connective tissue the cells had many points of resemblance with the cells of the infiltrate. According to Jordan the tumour consisted of naevus cells and of endotelionatous and neurinomatous tissue. In his opinion the corpuscles were made up of neurinomatous tissue, i. e. of Schwann-cells.

Brögli (1931). A boy of 16 with an extensive congenital pigmented area in the lumbar region. Eight months before extirpation the nodule in the pigmented area had started growing with ensuing spontaneous and pressure pains. The tumour proved to be a plexiform neuroma with innumerable formations resembling tactile end-organs. The corpuscles were partly solitary, partly disposed in small conglomerates. Their mean size was 190×100 microns and they were enclosed in thin capsules. The inner part of the corpuscle stained striped and reddish yellow in v. Gieson's solution. The nuclei were small, dark, oblong, and situated as a rule near the edge of the corpuscles. A medullated nerve-fibre was often seen to enter the corpuscle and its sheath continued into the capsule. Staining with Bielschowsky's method revealed thin, black neurofibrils in all the corpuscles. The corpuscles resembled at some places Vater-Pacini corpuscles, at other places Wagner-Meissner corpuscles.

Scherer (1934). A man 53 years of age having a large number of small skin tumours, a gigantic skin sac in the lumbar region and extensive small-

spotted pigmentation. It was diagnosed as neurofibromatosis. The tumour was characterized by the presence of large corpuscular conglomerates. A thin connective tissue capsule and septa extending from this capsule divided the area into many ovalshaped corpuscles of almost equal size and of fibrillar structure. The thin fibrils ran like meridians and stained greenish yellow in v. Gieson's solution. A few collagenous fibres were also encountered. The nuclei were relatively large, round oval in form, situated as a rule peripherally and chromatin-deficient. In some operations Scherer was able to follow quite closely and clearly the transformation of a nerve into a conglomerate of this kind. The Schwann-cells drew away from each other and in the interspace they gave rise to the above-mentioned fine fibrillar tissue, which was divided into areas of equal size.

Cleuet & Ingeltrans. A case of Recklinghausen's disease. A special feature were the small, oval or sausage-like formations staining yellow in v. Gieson's solution, having lamellar structure and resembling at some places Meissner's, at other places Pacinian corpuscles. Cleuet & Ingeltrans called the corpuscles »névrome type pseudopacinien et névrome type pseudomeissnerien«. In their opinion the corpuscles had developed from the perineurium.

Bailey & Herrmann (1938). A girl 13 years of age. 18 months before autopsy she had different attacks of paralysis. Autopsy revealed multiple nerve tumours. Referring to an oculomotor tumour Bailey and Herrmann give a description of some peculiar verticillated formations, clearly similar to Vater-Pacini corpuscles. In the middle of the corpuscles was a special cell, the cytoplasm of which stained yellow in v. Gieson's solution. A great many, perhaps all of them, were grouped round the medullated nerve. The gradual transformation of these corpuscular areas into areas with the classic neurinomatous structure could be followed. According to Bailey & Herrmann the corpuscles as well as the neurinoma tissue consist of connective tissue.

Cammermeyer (1946). A congenital tumour, removed from the finger of a man 50 years old. It contained connective tissue and small noduli which were surrounded by a capsule of collagenous fibrils. The septa extending from the capsule divided the nodules into round corpuscles, the number of which varied from 3 up to 331. Their mean size was $43 \mu \times 26 \mu$. The nuclei resembled Schwann-cells; in the corpuscles and between them numerous nervefibres were seen. It was diagnosed as tumour of tactile end-organs.

In addition to these I report now three new cases, which probably belong to the same group.*)

Case I.

A 36 years old farmer's daughter. If we except the tumour formation she had always enjoyed good health. On the left side of the patient's skull there had been a tumour ever since her earliest childhood. According to her mother's report the tumour had developed from a penny-sized naevus. She was operated on for the first time at the

*) Case I is from the Institute of Radiotherapy, Helsinki; case II from the »Radiumhemmet« in Stockholm and case III from Doctor Gellerstedt in Uppsala, who placed his notes at my disposal after my lecture in Uppsala in July, 1947.

age of seven. Another attempt to remove the tumour was made seven years later but it failed. After this the tumour had remained on the whole unchanged, with a tendency to flow downwards. The patient never complained of pains or tenderness in the tumour.

On examination the skin was found to be dry with a large number of freckles in the arms and legs. On the left side of the skull there



Fig. 1.

Case 1. The tumour just before the operation.

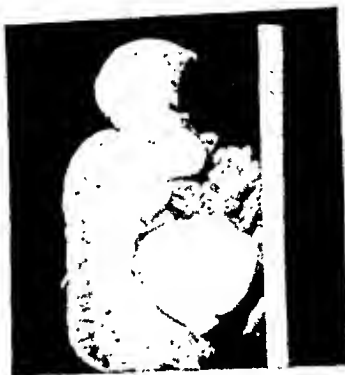


Fig. 2.

Case 1. Longitudinal section of the tumour.

was large tumour the size of a big loaf, adhering to the surface of the left temple, the cheek and the parietal region (Fig. 1). It extended downwards to the level of the lower edge of the jaw-bone. The left eyelid was almost closed and the left ear was completely deformed. The skin of the swelling was at places scabby or pigmented. Large bloodvessels and cavities made the extirpation of the tumour difficult and it proved impossible to have everything removed. The wounds healed well and about 18 months later the patient was admitted to hospital for a plastic operation. The treatment is still going on.

The cut surface of the tumour was light gray, fibrous and a little yellowish here and there. Parallel with and about 0.5 cm. from the surface ran a stripe, 40—50 mm. thick, brownish and with indistinct contours. Thus the cut surface had some points of resemblance with the cerebrum. Some transverse sections of large bloodvessels were seen. In the middle of the tumour there was a considerably harder, elastic, disk-like, sharply-defined formation, 6 cm. in diameter and 4 cm. thick at the centre (Fig. 2).

The samples taken from various parts of the tumour for microscopical analysis were all characterized by the presence of peculiar corpuscular formations. Otherwise they differed a great deal from each other. The epidermis was thickened throughout, hairs, sebaceous glands and glands which resembled salivary glands were seen here and there. Beneath the epidermis was a layer of connective tissue, varying in thickness and separating the tumour tissue from the epidermis. In low-power microscopic examination the tumour tissue seemed to consist of corpuscles of unequal size, which in some places were placed rather apart from each other and separated by connective tissue, while in other places they were congregated in close proximity, forming coherent areas and stripes, which in the neighbourhood of the epidermis lost their typical corpuscular structure (Fig. 3). A yellowish

tone was peculiar to the tumour tissue in v. Gieson's solution. Still, the tinctorial properties of the different parts of the tumour varied markedly. In Masson's trichrome stain the picture was identical, the corpuscles taking often a clear red tone. Transverse sections of nerves and blood-vessels of varying size were seen in the tumour area. Their structure appeared to be normal

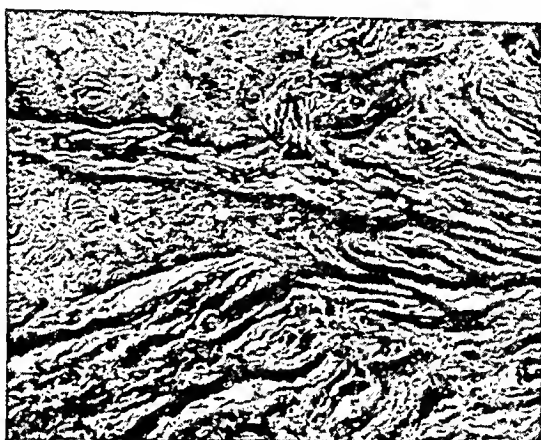


Fig. 3.

Case 1. The corpuscles form large areas and stripes which in the neighbourhood of the epidermis lose their typical corpuscular structure.
Masson's Trichrom stain.

and they seemed to have no connection with the corpuscular tissue of the tumour. Even a low-power microscopic examination of the tumour area revealed groups of cells with large and light-coloured nuclei.

The structure of the single corpuscle varied greatly in different parts of the tumour. In some instances they lay apart from each other, in other cases they were congregated in a string-like or rosette-like manner. Here and there they formed wide coherent areas or stripes. The corpuscles were round oval, 15—30 microns in breadth. They were clearly encapsulated and resembled, roughly, Wagner-Meissner corpuscles. In some instances the corpuscles were more homogeneous and compact, staining almost evenly violet in Masson's trichrome stain, and yellowish in v. Gieson's stain (Fig. 6). Red streaks were



Fig. 4.

Case 1. The corpuscles are at places side by side. Trichrom stain.

seen in v. Gieson's stain. The corpuscle had a shrunken appearance, as it were, and there was an empty space between the capsule and the surrounding connective tissue. The staining of the connective tissue fibrils revealed only a few fine fibrils in the corpuscles. The corpuscles in these areas lay in most instances apart from each other. In other places the lamellar structure of

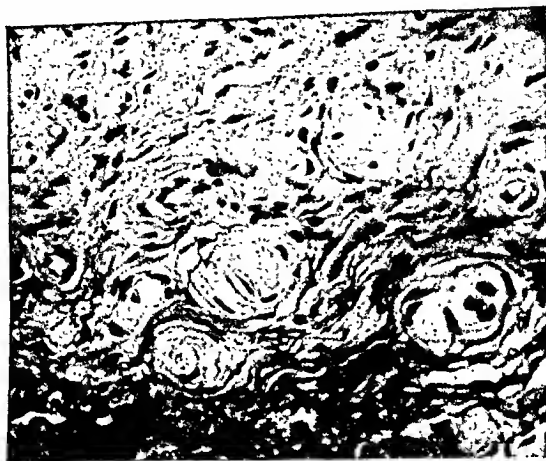


Fig. 5.

Case 1. The corpuscles are at places apart from each other and separated by connective tissue. On the right side larger, lighter-coloured cells of the corpuscles are visible. Trichrom stain.

the corpuscles was more clearly seen. They were larger and their fibrils stained reddish in Masson's stain, the intermediate substance taking the light red tone. The fibrils stained yellowish in v. Gieson's solution. In the stainings of the fibrils (Gömöri) the relatively thick capsule and the thin septa extending from it were clearly visible (Fig. 5). In some places the intermediate substance was absent and only the septa were seen, thick, staining yellow

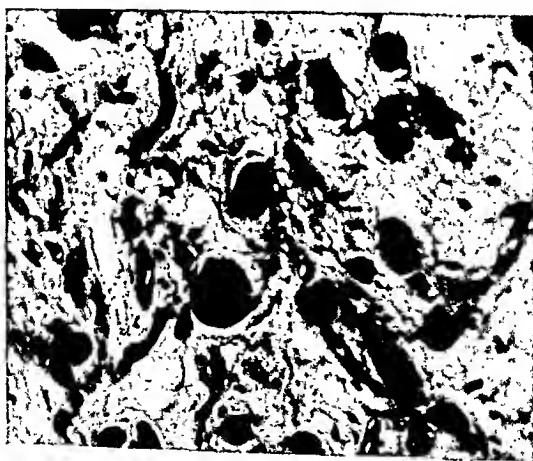


Fig. 6.

Case 1. An area with smaller and slightly homogenous corpuscles. The striped character is clearly visible in some corpuscles. v. Gieson stain.

red in v. Gieson's stain and blue in Masson's trichrom stain. The nuclei of the corpuscles were relatively small, dark, about the size of 3×6 microns, situated in most instances at the periphery and only rarely in the lamellae. One had an impression as if these nuclei belonged to the capsule and further, that the majority of the corpuscles contained bigger and lighter-coloured nuclei, about the size of 6×8 microns (Fig. 5). The boundary lines between the cells were not clearly seen in the corpuscles.

In the fat stains also the tumour tissue was beautifully distinguished from the subepidermic connective tissue, which remained colourless while the corpuscular areas stain slightly blue with Nile blue sulfate and reddish with scarlet red. Plenty of fatty tissue, often arranged in the form of stripes, was seen in the tumour.

In the stains of the nervefibrils (Bodian, Davenport) the axis-cylinders stained beautifully and looked quite normal. In the corpuscles no nervefibres were observed.

In the myelin stains no nerves were visible in the corpuscles but the corpuscular tissue was distinctly outlined and stained slightly grey while the connective tissue remained yellowish.

Case II.

A girl 8 years of age with an extensive pigmented area covering the middle and lower parts of the back. She had always been in good health and her development had been normal. About two years before she consulted a doctor her mother discovered a few small protrusions in this area. On examination a yellowish brown pigmentation was seen in the middle and lower regions of the back, with long, soft and white hairs. Numerous loosely fixed about pea-sized nodes of hard consistence were found. The skin covering them was loose and they were not tender. Some of them seemed to be confluent. The patient has been under observation for three years and her condition has remained unchanged. She had no subjective pains.

The samples removed from the nodules for histologic examination showed that the structure of the tumour was of the same type throughout. The

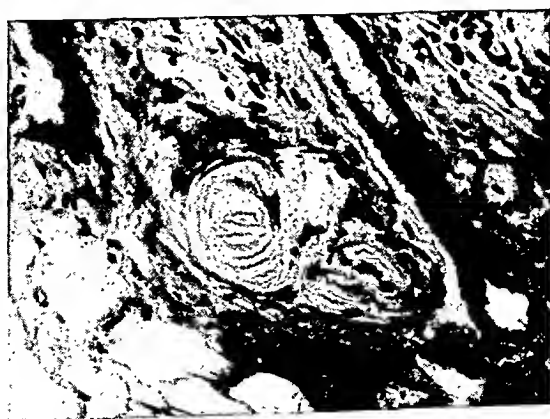


Fig. 7.

Case 2. A small corpuscle with lamellar structure. Trichrom stain.

epidermis and the subepidermic connective tissue seemed to be normal. A low-power microscopic examination of the deeper lying subcutaneous fatty tissue and of the loose connective tissue revealed thicker and thinner, irregularly shaped, encapsulated nerve-like stripes, some encapsulated tumours resembling loose fibromas, small (and a few bigger) corpuscles, which stained yellowish in v. Gieson's solution. It revealed, further, some areas with in-



Fig. 8.

Case 2. A nodule similar to a tactile corpuscle. The septa extending from the capsule are clearly visible. Gömöri stain.

distinct contours having large and light-coloured nuclei, which stained also yellowish. In the following I shall deal with these special features separately.

The nerve-like stripes. They ran in loose connective tissue, varying in size from quite thin ones to those 1 mm. thick. The stripes were clearly encapsulated, the capsule containing a large number of collagenous fibres. They consisted of very loose tissue with small, dark, oval nuclei about the size of $4 \times 6\frac{1}{2}$ microns. A few bigger light-coloured nuclei were also present. The tissue was rich in capillaries and fibres of considerable thickness which took the blue tone of the Masson stain. Further, some medullated nerve-

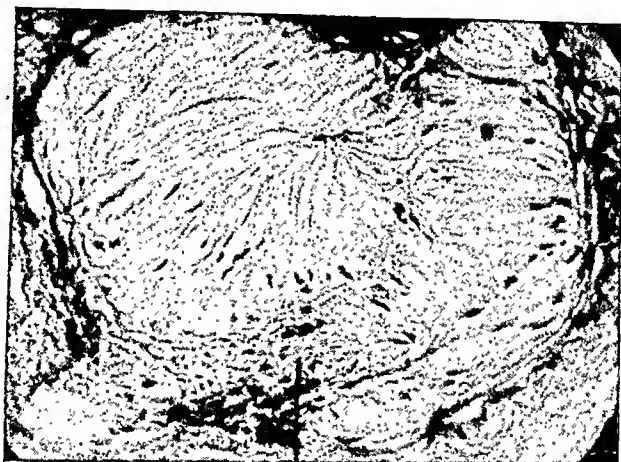


Fig. 9.

Case 2. A large corpuscle resembling a Vater-Pacini corpuscle. The inner bulb marked by strong hypertrophy. Trichrom stain.

fibres were also encountered, the fibres running usually in bundles in the middle of the stripes. The fibres were, however, rare and did not occur in all stripes. Some light-coloured nuclei the size of $6 \times 9 \mu$ and possibly interpretable as nuclei of Schwann-cells were seen in the stripes. There was, however, in the proper sense of the word, no increase of Schwann-cells. A description of the corpuscles found in some stripes will be given later in this article. In the staining of the nervefibrils (Davenport) spiral, black-impregnated fibril fragments were seen running in different directions in the loose tissue of the stripes, interpretable as nonmyelinated nervefibres. It was, then, a case of plexiform neuromaformation.

The tissue resembling *loose fibromas* was greatly similar to that seen in the stripes. No medullated fibres, however, were seen in it. The nuclei were in most instances small and dark, the size of $4 \times 6\frac{1}{2} \mu$, but in addition to these also larger and light-coloured nuclei were encountered. Fibres, about



A

Fig. 10.

Case 2. The centre of a large corpuscle showing the cylindrical structure.
A. Longitudinal sections of the cylinders. Gömöri stain.

2 microns in breadth, staining red and surrounded by a blue mantle, were seen in the oedema-areas. One had the impression as if all the thicker blue staining fibres were composed of cylinders of precisely this type.

There was a large number of *corpuscles* in the loose and very vascular connective tissue as well as in the fatty tissue. They varied in size from 20 up to 200 microns, the average size being about 40 microns. They occurred single or in larger conglomerates of 2–15 corpuscles (Fig. 7 and 8). The corpuscles were surrounded by a clear capsule of connective tissue. The fibril stain (Gömöri) revealed 1 to 5, in the larger capsules even 30 layers. The layers appeared to be often separated from each other by oedema-fluid (Fig. 9). The majority of the corpuscles had a clear lamellar structure, the fibres running like meridians from one pole to another and taking the form of concentric circles. They often had many points of resemblance with the Wagner-Meissner corpuscles. The structure of the larger corpuscles was obviously not lamellar. They consisted of cylinders, the interior of which stained blue in Masson's stain. The cylinders were surrounded by a thin blue mantle. Their transverse section was circular, varying greatly in diameter, which was greatest at the level of the oval nucleus (Fig. 10). The inner part of the corpuscles looked

homogeneous and stained yellowish in v. Gieson's stain, violet in Masson's stain. The tinctorial properties resembled greatly those of neurinoma tissue. The nuclei were generally situated at the periphery of the corpuscles. Their size was $4\frac{1}{2} \times 7 \mu$ and their longitudinal axis ran in most instances parallel with the capsule. Nuclei were found only rarely at the centre of the corpuscle. In the nervefibril stains no nervefibres were seen, but owing to the lamellar structure of the corpuscles the adjustment of the screw of the micrometer caused some light to reflect from the edge of the corpuscles and this light made the lamellae black. In some nerve-like stripes the medullated nerve was surrounded by concentric formations, which regarding their tinctorial properties and structure had a strong resemblance to the above-mentioned corpuscles.

In addition to these corpuscles the sample contained areas characterized by the absence of corpuscular structure but having the same tinctorial properties as the corpuscles. The nuclei of the areas were large, oval and light-coloured, resembling naevus cells.

Case III.

A man 17 years of age who had earlier been in good health. He had a congenital slowly growing swelling at the back of the skull. In recent times the swelling had grown more rapidly, the patient had headache and consulted a doctor.

On examination a large, soft resistant formation was found at the back of the head, moving on its foundation but intimately fixed to the skin. The whole formation was about 10×15 cm., hanging downwards. He was operated on and all the soft parts of the swelling, including the skin were seen to be thickened. The bone beneath seemed to have undergone no pathological change.

The wound healed by first intention. It has been followed for one month.

The samples taken from different parts of the tumour for histological examination were all more or less alike. The epidermis and the connective tissue beneath it were thickened. The connective tissue shaded imperceptibly and with no clear boundary line into tumour tissue, which stained yellowish in v. Gieson's solution and a light violet in Masson's trichrome stain. The tumour tissue was in some places made up of small corpuscles with lamellar structure, occurring sometimes single in the connective tissue, sometimes in string-like formations.

In some places the corpuscular areas shaded without any clear boundary line into areas without any clear corpuscular structure. These areas were, however, owing to their cellular structure and general tinctorial properties, of similar tissue as the corpuscles and differed distinctly from normal connective tissue. The nuclei were large, oval, about 5×7 microns, chromatin-deficient and lying as a rule at the periphery of the corpuscles. In the stainings of the connective tissue fibrils (Gömöri) the lamellae of the corpuscles stood out beautifully, being black-impregnated. The fat stains, nerve stains and myelin stains gave results which corresponded exactly to those in Case I.

How are we to interpret these single cases of tumour with corpuscular elements? Are the corpuscles really comparable to tactile

end-organs and are they made up of Schwann-cells? Are the corpuscles mere sporadic findings or do these tumours form a coherent whole? Are these tumours associated with melanomas, neurinomas or with v. Recklinghausen's disease complex?

I mentioned above that the corpuscles of the tumours often resembled Wagner-Meissner corpuscles. A normal Wagner-Meissner corpuscle is about 80×30 microns in size and is situated as a rule immediately beneath the epithelium. It is surrounded by a connective tissue capsule from which septa of varying thickness and transverse to the axis extend. The inner part of the corpuscle consists of oval or pearlike cells lying vertical to the longitudinal axis and their nuclear pole looking toward the periphery. The majority of the corpuscles of the tumours in question were equal in size. A capsule and septa extending from it were also distinguishable. It is difficult to conclude, on mere morphological grounds, whether their inner part is made up of Schwann cells, particularly so, as there is, to my knowledge, no specific staining method for Schwann cells. The corpuscles differ considerably from connective tissue in that they stain yellowish in v. Gieson's solution and reddish in Masson's trichrome stain, having thus many points of resemblance with neurinoma tissue. The nuclei differed also from those of common connective tissue cells and were easily interpretable as nuclei of Schwann cells. The big corpuscles of the tumour of the lumbar region (case II) can only with difficulty be included in any group of tactile corpuscles. What they resemble more than anything else are the Vater-Pacini lamellar corpuscles, which as we know are large, oval corpuscles, 0.2—2 mm. in length, consisting of a medullated nerve fibre, an inner bulb and of connective tissue lamellae. This might be regarded, then, as a case of a violent enlargement of the inner bulb in the corpuscles of the tumours.

There are previous reports on corpuscles of tumours. In six cases they were supposed to consist of Schwann cells, in one case to have originated in the perineurium and in one further case to contain connective tissue. Flörcken & Steinbiss held the view that the corpuscles have resulted from the productive growth of the Schwann cells of the peripheral nerves. Masson and Jordan, in reporting corpuscles on the scalp, concurred to this view. According to the former, the structure of the corpuscles corresponds to that of the supporting apparatus of the Wagner-Meissner corpuscles. Jordan thinks the corpuscles contain neurinomatous tissue, i. e. they consist of Schwann cells. Brögli, Scherer and Cammermeyer agree with this view that the corpuscles are made up of Schwann cells. They have also been able to discover nervefibrils in them.

In view of the tinctorial properties of the corpuscles which differ from those of connective tissue and in view of the structure of the nuclei, I am inclined to believe that the corpuscles consist of Schwann cells.

What is the relationship, then, between these tumours and the *neurinomas*, which also, as I pointed out above, contain corpuscular structures?

No attempt will be made here to discuss in detail the subject of *neurinomas*. I just mention briefly that according to some authorities they are ectodermal tumours, composed of Schwann cells, Schwannomas, while others favour the view that they consist of connective tissue and call them perineurial fibroblastomas.

My material consists of 60 cases of pure peripheral subcutaneous *neurinoma*.*)

In a great many of them I have been able to discover different nodular and corpuscular formations which often resemble greatly Wagner-Meissner tactile corpuscles. I am inclined to favour the view that the *neurinomatous* tissue is made up of Schwann cells. From the point of view of tinctorial properties, and cellular structure these corpuscles present a picture identical with that of *neurinomatous* tissue. They are, then, in my view, also made up of Schwann cells. According to Masson these palisaded nodules of the *neurinomas* are, from the point of view of histogenesis and structure, comparable to the supporting apparatus of the Wagner-Meissner corpuscles. In view of the fact that palisaded nodules are not found in all varieties of *neurinoma*, Masson suggested the possibility that the Schwann cells may be in some way affected by the motor and sensory neurites and that palisaded nodules consequently occurred only in sensory nerves. Murray & Stout (1940) base their similar views on the evidence of tissue culture.

The palisaded nodules of *neurinomas* and the corpuscles of the corpuscular tumours — which are under discussion here — are comparable to the Wagner-Meissner corpuscles (Masson). I was not able, however, to distinguish any clear capsule or septa in the palisaded nodules of the *neurinomas*. It seemed to be rather a case of a restricted tissue area, standing out in distinct outline from the surrounding tissue. All the staining methods employed by me revealed similar tinctorial properties in both types of corpuscle. In neither case were the cells typical connective tissue cells. They had more points of resemblance with Schwann cells. In case III the corpuscular areas were seen to transform into areas resembling *neurinomatous* tissue. It strikes me as not unlikely that the palisaded nodules of the *neurinomas* and the corpuscles of the tumours under discussion are comparable with each other.

Also the *pigmented naevi* and these tumours seem to have certain features in common. Masson, whom I quote extensively, thinks that the corpuscles found in *naevi* and the Wagner-Meissner end-organs are analogous formations. He published a report on a tumour of the scalp and interpreted it as a *pigmented naevus* or rather as a *neuro-*

*) The majority of cases are from Professor Reuterwall's private material.

naevus, which differed from the common type of naevi in that it had a deeper lying plexiform neurinoma formation and large tactile corpuscles. Jordan gives a similar interpretation of a case of tumour. In the cases recorded by me the scalp tumour had developed from a dark birth mark and the other tumour was situated in a wide pigmented area. In both tumours there were corpuscles and conglomerates of naevus cells side by side. The view that these tumours and the pigmented naevi may have something in common is supported by the fact that pigmentation was absent in only one of the previously published cases.

As to the *clinical aspects* of these tumours we see that apart from the cases reported by Bailey & Herrmann and by Cammermeyer both differing in structure from the rest of the tumours discussed here, all of them were situated either on the scalp or in the lumbar region, which are the favourite sites of plexiform neuroma, as has been demonstrated by Bruns and Strauss on the basis of a material of 46 cases and 80 cases respectively. The age of the patients varied from 3 up to 55, and the tumours were undoubtedly congenital in 5 cases. The size of the tumours varied from little finger's end to those the size of a child's skull. In 4 cases the tumours were in the form of Cutis verticis gyrata. The subjective pains of the patients were insignificant if we except the inconveniences arising from the size and position of the tumours. Ache was present in but 2 cases. Reliable evidence of absence of pigmentation was supplied only in one case. As regards the distribution of the pigmentation even other parts of the body surface may be involved. General neurofibromatosis developed in two cases. From the clinical point of view all the tumours were benign.

As a *summary* of the 11 cases the following facts may be recorded. Eight of the tumours were situated in the lumbar region or on the scalp. In 4 cases the tumours had a tendency to hang in folds and they were, then, in the form of Cutis verticis gyrata. The majority of the patients were young and in 5 cases the tumour was congenital. From the clinical and histological point of view all the tumours were benign. Pigmentation was absent in only one case. General neurofibromatosis developed in two cases, and in 3 cases a plexiform neuroma occurred. Nerves were found in all the tumours but the corpuscles were innervated in but three cases. Even in these three cases the evidence was not quite convincing. All the corpuscles stained yellowish in v. Gieson's solution and all had lamellar structure. The corpuscles of the neurinomas, the pigmented naevi and of the tumours under discussion have a great many points of resemblance with each other. They all resemble Wagner-Meissner tactile end-organs more than anything else.

The tumours in question are obviously corpuscular tumours be-

longing to the still rather indistinct group of pigmented naevi, neurinomas, neurofibromas and plexiform neuromas. They certainly have some connection with nervous elements.

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NO DISCUSSION

EXPERIMENTAL GENETIC STUDIES ON LEUKEMIA IN MICE

By *Georg Hogreffe*.

The heredity of spontaneous leukemia and the susceptibility of mice to transplanted leukemia have already been investigated extensively, among others, by *MacDowell* and collaborators and by *Furth* and collaborators in U. S. A. Originally the experiments of which a preliminary account will be given here were merely aimed as a reproduction of these previous investigations. In the course of the present experiments, however, a number of interesting details were met which, I think, deserve particular mention.

Our material consists of two mouse strains — one with a very high frequency of spontaneous leukemia, the other with a very low frequency of this lesion — together with a number of hybrid generations resulting from crossing between the two strains.

The Aka strain has been inbred through many generations, and originally it was brought to Denmark from *Furth's* Laboratory in U. S. A. About 60 % of these mice, females as well as males, die of leukemia at the age of 6—16 months, most often at the age of 9—10 months. As a rule the affected animals show a very immature cell type, less frequently a state of leukemia that may be classified as lymphogenous or myelogenous. In about 28 % of the cases the disease manifests itself merely as an enormous enlargement of the thymus, while in 42 % a marked enlargement of the thymus is accompanied by universal enlargement of the lymph nodes together with varying changes in the liver and spleen. In the remaining 30 % of the positive cases the picture of the lesion is characterized by universal leukemic changes without any particular enlargement of the thymus.

The leukemia-free strain, which we have designated as B, is not quite entirely inbred. Still, breeding between sibs has been so frequent, and the individuals employed for the breeding have been so near re-

lated that it is safe to reckon with a fairly high degree of homozygosis. The mice have been bred in this institute through many years, and in all this time only one case of lymphogenous leukemia has been observed, besides two cases of bone tumors.

Now the two strains mentioned have been crossed in such a way as to give two different F_1 generations and two different F_2 generations, namely by mating Aka males with B females, and B males with Aka females. The purpose of this procedure has been to investigate the possible existence of a maternal or paternal factor that may be of significance to the development of the disease. Furthermore, from both F_1 generations, back-crossings to the original strains have been carried out.

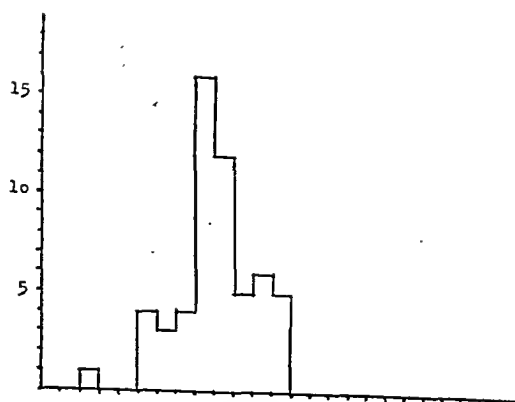
In order to investigate the inheritance of the spontaneous leukemia, we have allowed a number of mice from each generation to die spontaneously. A thorough autopsy has been performed on all the mice and, whenever practicable, microscopic preparations have been made. As a number of the animals are living yet it is not possible at this juncture to give any definite numerical data on the frequency of leukemia in the various generations. On the other hand, a graphic presentation of the age-classes in which the animals died of leukemia shows some very interesting features.

Fig. 1 shows a curve for the number of mice in the first filial generation that died of leukemia in the various age-classes, 60 days being chosen as the width of each class. Thus it is plainly evident that here the maximum number of deaths falls round the age class 480—540 days, in contrast to the Aka strain (see Fig. 5) in which the maximum falls round the age-class of 270—330 days. Considering the short average lifetime of the mice, this shift is rather considerable.

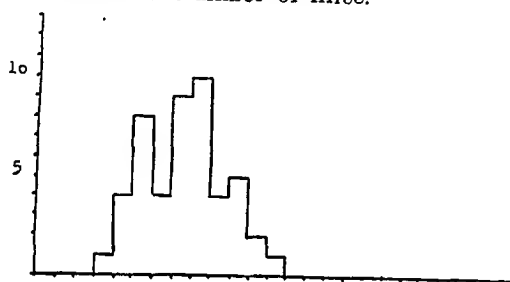
Fig. 2 shows a corresponding curve for the second filial generation. Here appears to be a tendency to two maxima: one round 300 days, *i. e.*, corresponding approximately to the maximum for the Aka strain; the second round 480—540 days, at which juncture we found the maximum for the first filial generation.

Back-crossing to the Aka strain (Fig. 3) likewise shows a notched curve, but here the number of mice dying of leukemia at a young age is relatively much greater than in the second filial generation.

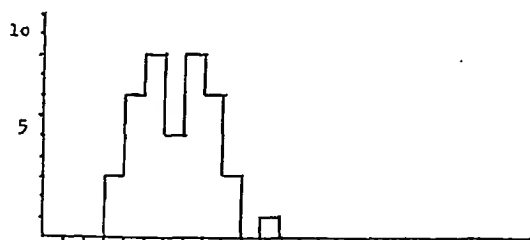
In back-crossing to the B strain the appearance of leukemia is relatively rare, on which account this curve (Fig. 4) represents merely a small number of mice (14), and this appears chiefly to be made up of late cases.

*Fig. 1.*

Age-distribution of 56 F1-mice died with leukemia.
 Abscissa: age in periods of 60 days.
 Ordinate: number of mice.

*Fig. 2.*

Age-distribution of 48 F2 mice died with leukemia.
 Explanation: see Fig. 1.

*Fig. 3.*

Age-distribution of 44 mice from the backcross F1 x Aka,
 died with leukemia.
 Explanation: see Fig. 1.

*Fig. 4.*

Age-distribution of 14 mice from the backcross F1 x B,
 died with leukemia.
 Explanation: see Fig. 1.

On addition of these four curves, resulting in a distribution curve for all the hybrids in this crossing, we have a markedly notched curve — as is plainly evident from Fig. 5.

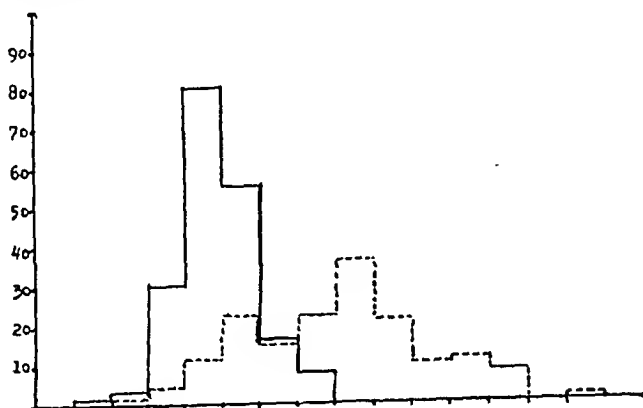


Fig. 5.

————— age-distribution of 193 mice from the Aka-strain died with leukemia.
 - - - - - age-distribution of 162 hybrid mice ($F_1 + F_2 + F_1 \times \text{aka} + F_1 \times B$) died with leukemia.

Explanation: see Fig. 1.

In Fig. 5, for the sake of comparison, an additional curve is plotted, representing the Aka mice that died of leukemia in the various age-classes. It will be noticed that the first maximum of the hybrid curve is identical with the maximum for the Aka mice, while the second maximum coincides with the maximum for the first filial generation (see Fig. 1).

Considering these curves, the idea obviously suggests itself that here we meet with a segregation in early and late cases of leukemia, dependent upon genotypic factors. It seems reasonable to assume the presence of one or more factors which homozygously contribute to an early development of the disease, whereas the heterozygous animals preferably acquire the disease later in life.

From the present material it further seems evident that the type of leukemia varies somewhat between the different generations. This is indicated by the data recorded in Table 1 on the frequency of clear-cut enlargement of the thymus as the only manifestation of leukemia in percentage of the total number of cases in each generation.

Table 1.
 Percental Frequency of Enlargement of the Thymus in the various Generations.

	Aka	B	F_1	F_2	$F_1 \times \text{Aka}$	$F_1 \times B$
Number of cases.....	200	0	56	48	44	14
Enlargement of thymus, %.....	28	0	7.1	10.4	25	0

From Table 1 it will be noticed that enlargement of the thymus occurs more frequently in generations with many early cases of leukemia, even though it would not be justifiable from this to draw any definite conclusion about the causal connection of this feature.

Also with regard to the distribution of myelogenous, lymphogenous and stem-cell leukemias there appears to be some variation between the different generations, as the incidence of decidedly myelogenous or lymphogenous leukemia among the late cases is higher in F_1 and F_2 than in the Aka strain.

Once the total material can be worked up, these problems will be dealt with more thoroughly and, among other things, by a statistical treatment of the material it will be investigated whether these phenomena may be explained in keeping with the prevailing laws of heredity.

Concurrently with these experiments we have investigated the inheritance of the susceptibility to transplanted leukemia. Thus, on a large number of mice from the Aka strain, B strain and the different hybrid generations of the crossing we have transplanted leukemic tissue from spontaneous cases of the disease in the Aka strain. We have not employed any single continuously transplanted line, but we have steadily used new spontaneous cases for the transplantation. On this account, of course, it will not be possible statistically to elucidate the inheritance of the susceptibility by the segregation in the 2' filial generation, as the inheritance of the susceptibility to leukemic cells from different lines may very well be somewhat divergent. In this way, on the other hand, the chance of mutations appearing in the transplant is reduced.

In practically all these cases the Aka mice showed 100 % takes, while the B strain was completely resistant. Also the first filial generation as well as the back-crossing from this to Aka showed 100 % takes, whereas the second filial generation on an average showed about 60 % takes. This is in keeping with all previous investigations, which shows that the susceptibility of the animals is dependent on the presence of one or more dominant genes common to the transplant and the host.

Our experiments have shown a new feature, however, namely: that the pathologic-anatomical picture resulting from the growth of the transplant is also dependent on genetic factors. In the Aka strain as well as in the first filial generation and in the back-crossing $F_1 \times$ Aka we always found the same picture: the transplant, which was minced before its intraperitoneal injection, gave rise to a morbid condition completely resembling the spontaneous universal leukemia with enlargement of peripheral and central lymph nodes as well as of the liver, spleen and, in some cases, the thymus too. In contrast hereto, a segregation was observed in the 2' filial generation: In a majority

of the cases — *i. e.*, in about 60 % — we found a take like that described in the Aka mice, but in about 20 % merely a localized growth of the injected cells was obtained, forming a sarcoma-like tumor, most often at the site of the transplantation but, in a few cases, also far way from this, *e. g.*, in the mediastinum. In the remaining cases (about 20 %) we found a condition intermediate between the generalized form and the tumor-like growth.

Among the offspring from the back-crossing $F_1 \times B$ we found only localized growth or the intermediate type.

From this it is evident that also the way of growth is dependent on genotypic factors. So the question arises at once: Which are the factors here involved? How do the factors here involved exert the action?

We have tried to find out whether the different forms of take might involve some difference in the degree of the differentiation of the leukemic cells. Among other things, we have measured the size of the nuclei in preparations from the tumors and from cases of universal leukemia developing from the same transplant, and in some cases the nuclei were found on an average to be larger in the localized form of the takes than in the corresponding generalized, indicating a lesser degree of differentiation in the former. Examinations of this kind are encumbered with many sources of error, however, and the results are to be accepted only with some degree of reservation, until a technique permitting a more exact estimation is available. At present we are able merely to establish the fact that genotypic variations between the cells of the transplant and of the host are decisive not only of the take of the transplant but also of the features of the pathologic-anatomical picture of the take.

Summary.

A brief account is given of studies on the age distribution of mice that died of leukemia, comprising an inbred strain with about 60 % leukemia and in different generations of a crossing between this strain and the leukemia-free strain.

From the findings it seems evident that the animals encumbered with the strongest hereditary taint die from the disease at a younger age than do the mice with a less pronounced taint. Further, also the type of leukemia is shown to be dependent on hereditary factors.

Finally, mention is made of a number of transplantation experiments showing that the pathologic-anatomical features appearing in this way are in part dependent upon hereditary factors.

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DISCUSSION

ENGELBRETH-HOLM: No abstract received.

G. HOGREFFE: It is to be pointed out that the pathologic-anatomical picture arising from transplantation of leukemia is quite uniform whether the transplanted originate from a clear-cut tumor of the thymus or from a generalized leukemia. This indicates that in the AKA strain the thymic tumor is no diseases *per se*, separate from leukemia in general, but merely a particular form of manifestation.

CYTOLOGICAL FINDINGS IN EXUDATES AND TRANSUDATES

By G. Wihman.

In 1936 the author introduced a new cytological method of investigation*) for pleural exudate and ascites which will be briefly recapitulated. The exudate is centrifuged, preferably in several large centrifuge tubes. The sediment is deposited in a small amount of water and transferred by means of a funnel into an ordinary raw sausage skin, approximately 1 dm long and drawn together at the lower end. This skin, which contains the concentrated exudate, is then placed in a centrifuge glass, partly filled with water, and its upper end is fastened to the opening of the glass by means of a rubber plug, as shown in Fig. 1.

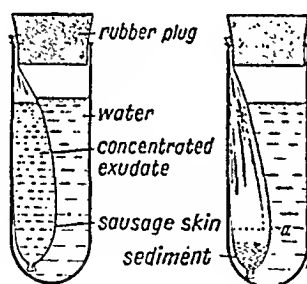


Fig. 1.

The concentrated exudate in the sausage skin is then centrifuged, then extracted and the skin tied together at a. The whole sediment is thus collected in the bottom part of the sausage skin and, since it consists of animal tissue, the whole of it can be submerged in the usual fixation and embedding fluids. After embedding in paraffin, the sediment can be cut like a common tissue piece and, further, the sausage skin can, at microscopy, easily be distinguished from its content. This is due to the loss of tissue structure of the intestinal

*) Wihman, G.: Klin. Wochenschr. 1936, 26, 926.

wall at the treatment undergone at the butcheries. (The raw sausage skins are kept in 1 per cent solution of formalin, when the salt has been washed away).

This method gave good results and it was soon found possible to simplify it further. After the first centrifugation, the fluid is not removed altogether but a small quantity is left ($0.1-0.3\text{ cm}^3$) above the sediment. The remainder is carefully stirred with a platinum rod. The concentrated sediment fluid is then poured through a funnel, at the lower end of which is fastened a sausage skin drawn together with a common Acwing-thread at the bottom (see fig. 2). The sausage skin is then tied in a knot above the sediment and the superfluous skin is cut off. The sediment contained in the sausage skin is dropped into a vessel holding a 10 per cent solution of formalin for fixation. The preparation is thereupon embedded in paraffin, like an ordinary tissue piece. It is appropriate to cut off both the knots immediately before dipping it in the paraffin.

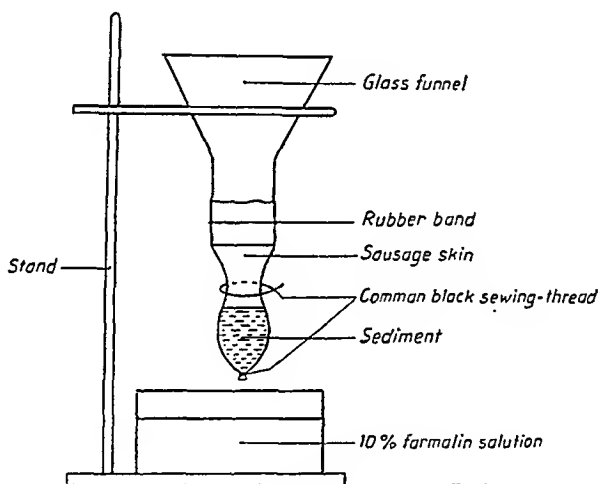


Fig. 2.

The results obtained by means of this simplified process are even better than those of the original method. By stirring the sediment, a more even and better distribution of the cellular elements is accomplished.

With this simplified method I have examined a large number of effusions and I will now with help of microphotographs try to give a description of the most important forms.

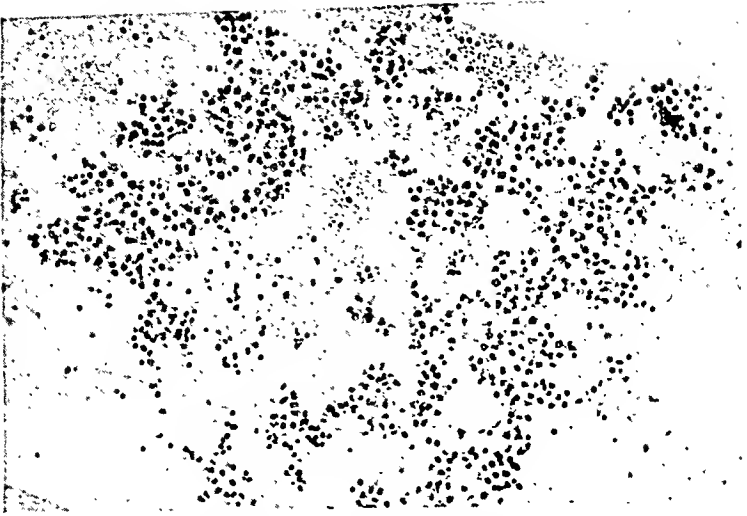


Fig. 3.

The *uncomplicated transudate* contains endothelial cells, endothelial aggregates and a moderate amount of lymphocytes and erythrocytes.

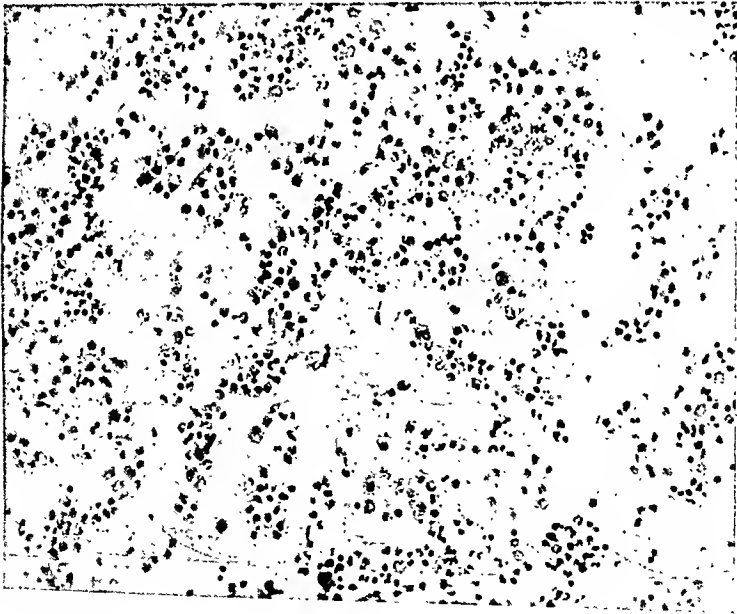


Fig. 4.

If a transudate is *complicated by a small pulmonary infarct* polynuclear cells also appear. The endothelial cells then also look swollen and are partly vacuolized.

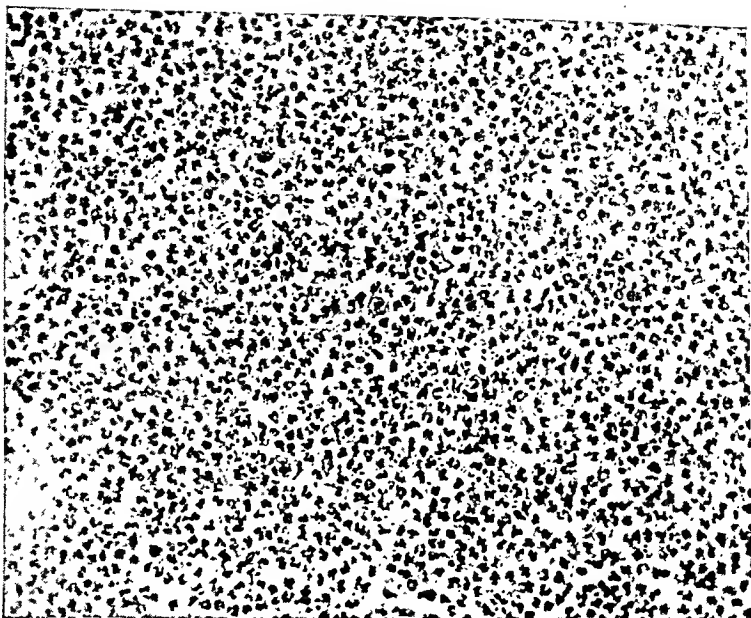


Fig. 5.

If a transudate is complicated with a large pulmonary infarct the number of leucocytes increases immensely and we get a picture that cannot, morphologically, be distinguished from nonspecific empyemata.



Fig. 6.

The *tuberculous pleurisy* in the initial stage shows leucocytosis. This stage is of little practical importance, so it will not be considered here. After some days a pronounced lymphocytosis appears and, in addition, fibrin appears between the cells.

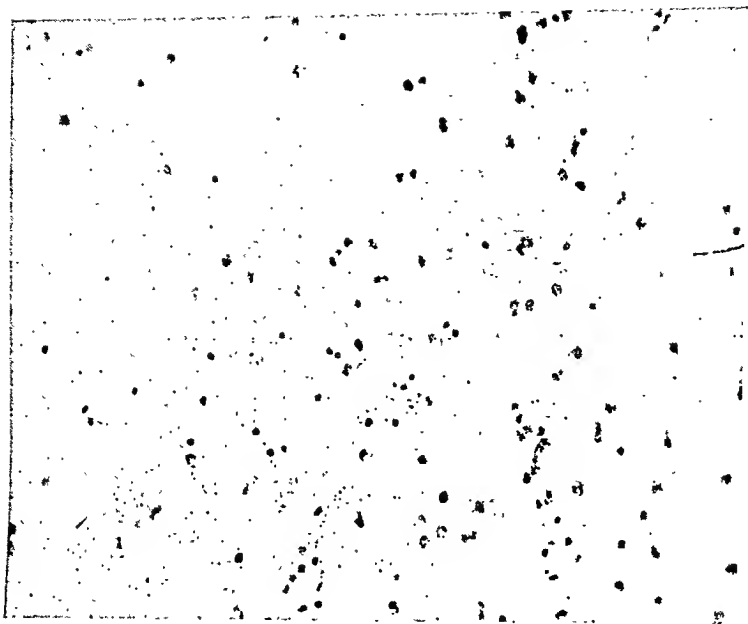


Fig. 7.

If the tuberculous pleurisy does not heal in the lymphocytotic stage described in fig. 5, and deterioration sets in, polynuclear cells again appear, the fibrin increases and becomes more coarsely fibred.

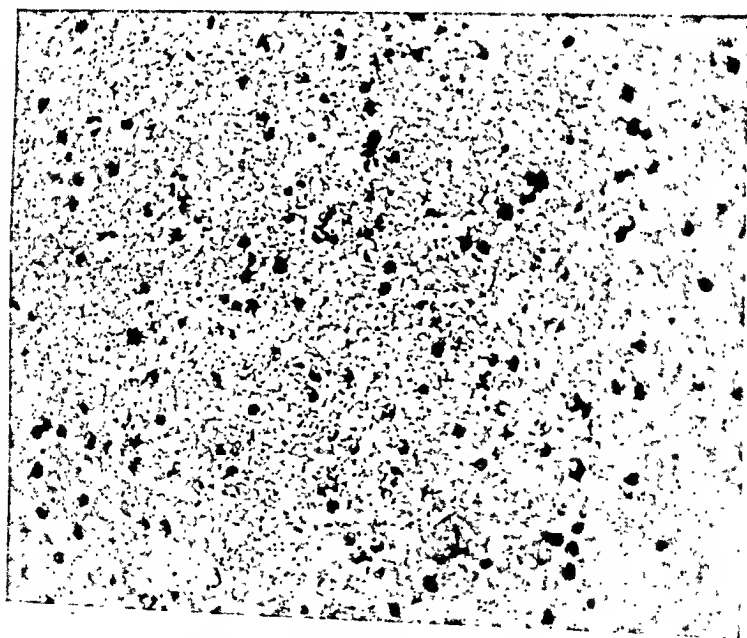


Fig. 8.

If the deterioration continues the pleurisy changes into the *tuberculous empyema* which shows decomposing leucocytes and endothelial cells and a more or less amorphous intercellular substance. To the largest part this probably consists of decomposing fibrin.

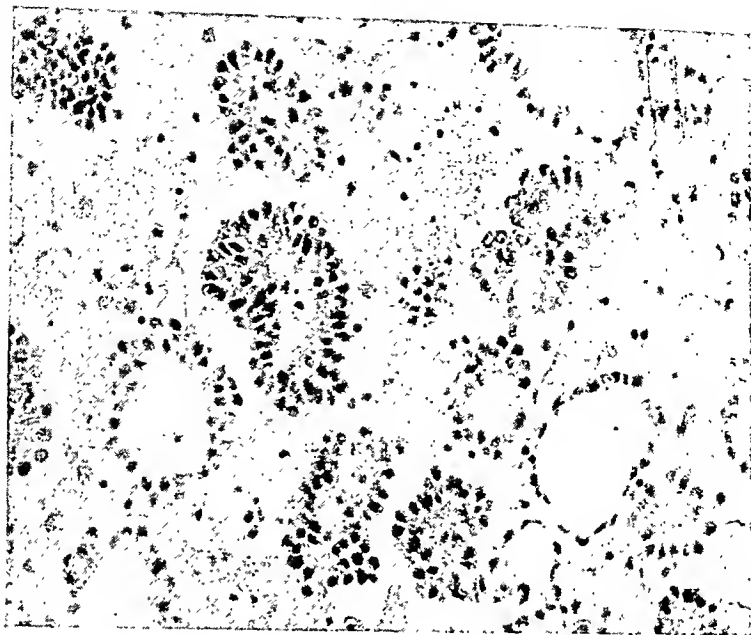


Fig. 9.

As an example of a *tumor exudate* here only will be shown a pleurisy in a case of bronchial cancer. Several pieces of tumor tissue were found in the sections from the sediment.

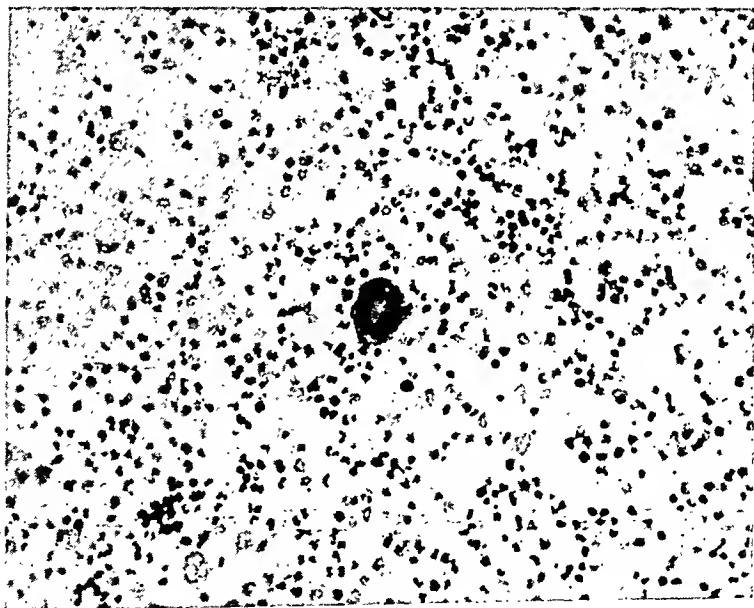


Fig. 10.

In cases of *rheumatic pleurisy* the sediment as a rule contains a large number of eosinophil leucocytes and large mononuclear cells of uncertain genesis. Sometimes one finds giant cells with 10—15 nuclei. They probably are giant cells of the same kind as are found in rheumatic granulomata.



Fig. 11.

In rare cases of *rheumatic pleurisy* also larger aggregations of cells are found. Their centre is in v. Gieson stain distinctly red. How these aggregations are formed is not fully known. Possibly, in severe cases of *rheumatic pleurisy*, excrescences are formed on the pleural surface looking like papillomata and of similar kind as in *rheumatic pericarditis* (see fig. 11) and in *rheumatic polyarthritis* (see fig. 12). If such excrescences fall off and are cut through, cellular aggregations of the above kind may possibly appear.



Fig. 12.

Pericarditis with numerous papillomatous excrescences in the healing stage (after E. Kaufmann).



Fig. 13.

Knee joint in a case of polyarthritis showing papillomatous excrescences in the synovialis (after H. Bergstrand).

DISCUSSION

LENNART SILVERSTOLPE: In connection with Dr. Wihman's paper, I wish to demonstrate a simple method for estimation of cells in body fluids. The method is based on centrifuging in a centrifuge tube of special construction by which the cells are centrifuged down in a sediment and separated in different layers according to their specific weight. This brings about a differentiation of the cells in the sediment: blood cells separate from epithelial cells and, as a rule, malignant cells from benign ones. The construction of the tube allows the sediment to be removed without disturbing the layers, so that after the histological treatment the cells keep their place in the layers — also when mounted on slides.

Technique: In the ward, to the specimen of fluid is added KBrO_3 to make 0.6 %. This stops the proteolytic processes in the cells, giving thus a preliminary fixation.

If the volume of the specimen is over 10 cc., it is placed in a tapering glass and left standing at a temperature of about 10° for 20 min. Then 10 cc. is transferred from the bottom of the glass to the above-mentioned centrifuge tube (see Fig. 1), which is turned upside-down a few times for the sake of equal distribution. Now one drop of Duboin's fluid is added (20 cc. saturated sublimate solution, 5 cc. glacial acetic acid, 1 cc. formalin) and allowed to sink to the bottom. A heavy white precipitate is formed on the bottom cork. If no precipitate appears (absence of albumin), 0.5 cc. serum is added to the contents of the tube, and, after shaking, a new drop of Duboin's fluid is added. Then centrifuging for 10 min. at a rate of 3500 revolutions per min. On removal of the bottom cork, the centrifugate comes along without any harm being done to it; and by placing a finger on the small glass tube that perforates the upper cork, the fluid will remain in the tube (see Fig. 1). The centrifugate, which readily is detached from the bottom stopper, is now transferred to Duboin's fluid, in which it undergoes the final fixation for $\frac{1}{2}$ hour, after this, the usual histological treatment, with embedding in paraffin. The blood is cut in sections in the longitudinal direction of the preparation.

The advantages offered by this method, besides its simplicity is a good arrangement and fixation of the cells. Through their difference in specific weight, the epithelial cells are kept separate from the blood. As a rule, also malignant cells are found in a layer other than that of benign cells, and even in specimens very rich in blood the epithelial cells will be separated from the blood cells. Now this method has been adopted as a routine method in the Radiopathological Institute of the Karolinska Hospital, Stockholm.

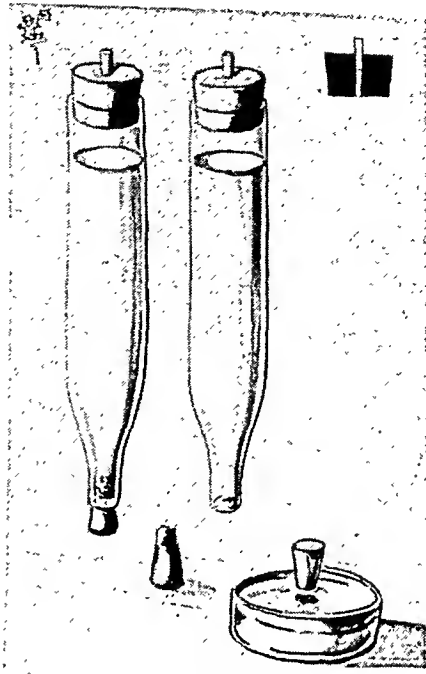


Fig. 1.

The tube, after centrifuging, with the centrifugate on the bottom cork, and its removal from the tube (the rest of the fluid remains in the tube). Final fixation in a dish containing Duboin's fluid.

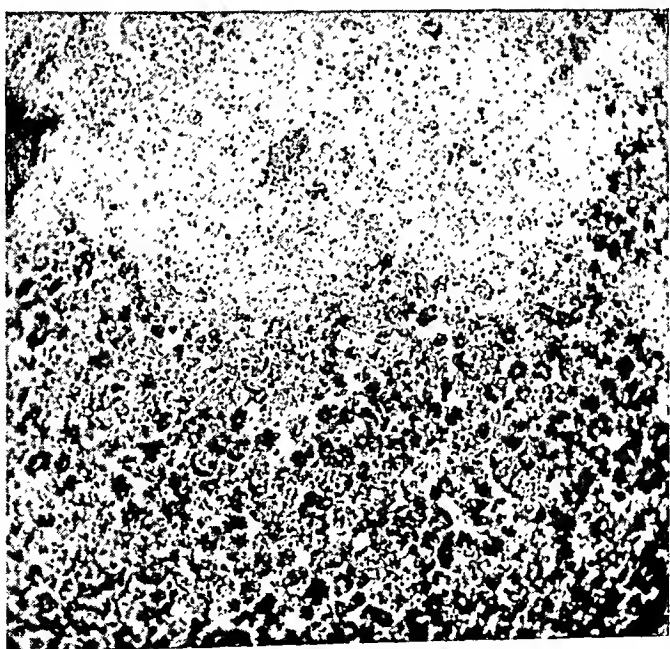


Fig. 2.

Separation of the cells in different layers.
 A = malignant cells.
 B = benign cells.
 C = red blood cells.
 D = white blood cells.

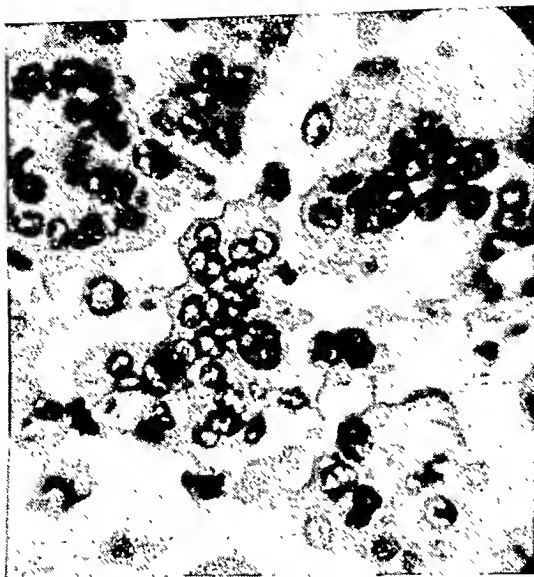


Fig. 3.

Magnification of the malignant cell layer stained with hematoxylin-eosin.

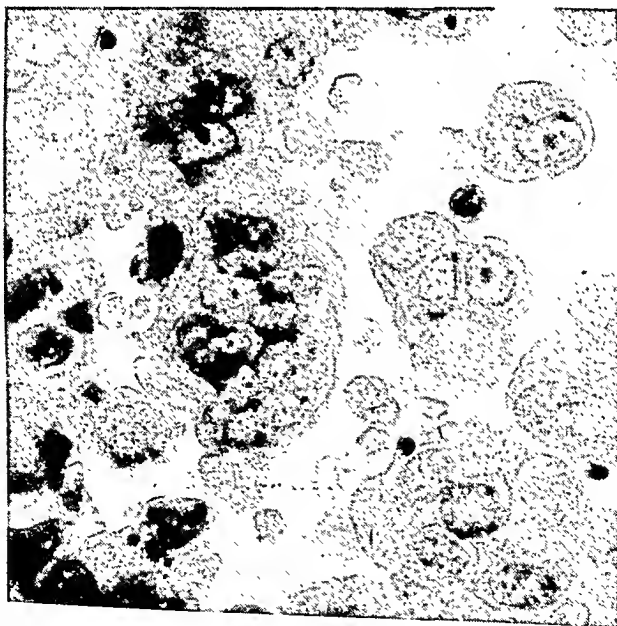


Fig. 4.

Same cells as in Fig. 3, photographed in ultraviolet light at 2.600 Å after the Caspersson method.

HYPERTENSIVE VASCULAR CHANGES IN THE EYE

By *Hilding Bergstrand*.

In 1929, Volhard suggested that those changes in the fundi which can be observed in cases of glomerulonephritis should be called *angio-spastic retinitis* instead of *albuminuric retinitis*, as earlier. It was his opinion that such changes are brought about by contraction of the retinal vessels and are due to hypoxaemia. This should also hold good in eclampsia and in essential hypertension with or without renal lesions. He opposed the former theory that retinitis was caused by some toxic substance.

Volhard's theory has, to a great extent, been accepted, with the exception of a number of debatable points. Thus, Friedenwold (1930) is of the opinion that retinitis in essential hypertension is always due to organic changes in the vessels, and he states that he has never seen any concentrations in the arteries. Furthermore, it is clear that all changes can not be regarded as a result of hypoxaemia. This is particularly applicable to the papillary stasis which is so characteristic of essential hypertension.

Volhard's theory has the great advantage that it gives a common explanation of the similar changes in diseases as dissimilar as eclampsia, chronic glomerulonephritis and essential hypertension with or without renal disorders.

As regards eclampsia, it is clearly indicated that contraction of the vessels can be the cause of retinitis. It has been observed how in young, previously completely healthy women the eclamptic attack is accompanied by a more or less pronounced narrowing of the arteries of the retina and the appearance of exudate and haemorrhages. The lesions are regressive and can disappear entirely after parturition. This has been demonstrated by Mylius by means of photographs of the fundi taken before and after parturition. Such changes can at times be observed without concurrent clinical symptoms from the kidneys.

Contraction of the arterioles of the fundi has also been observed in glomerulonephritis. Wagener is even of the opinion that it is pos-

sible to differentiate between albuminuric retinitis in glomerulonephritis and in essential hypertension, since in the former sclerosis of the vessels is not present. In glomerulonephritis it would thus always be a contraction. Whether this is correct or not, I am not in a position to assert, since I have had insufficient material at my disposal to investigate the question.

Statements in the literature on this matter are very vague. Garsteiger says, however, in 1937, that he had found fatty deposits in the vessels of the retina in glomerulonephritis, but he gives no clinical histories which make it possible to ascertain the diagnosis. A number of the patients were, however, very young.

As mentioned above, the cause of the changes in essential hypertension is the subject of controversy. A number of authors, however, state definitely that they have observed localized narrowings of reversible nature in the arterioles, which they consider to be due to contractions. Gans in 1944 thought it is possible to differentiate between a contraction and an organic lesion in essential hypertension by means of the appearance of the arterial reflexes. I shall return to this question of reflexes later.

Before I describe the changes in the vessels, I must say a few words concerning terminology. As it is known, the central artery of the retina divides at the optic disc, or just before it, into a superior and an inferior branch which in turn divide, on the surface of the disc, into a temporal and a nasal branch. In the textbooks of anatomy, these branches are called respectively arteriola superior and inferior, temporalis et nasalis. Their structure is that of arterioles, with very few muscle fibres in the walls, despite their somewhat considerable width. Friedenwold, however, wishes to call the above mentioned vessels arteries up to their first division, and thereafter arterioles. For the sake of convenience, in this paper, all these vessels will be referred to as arteries.

In the examination of the eyes of patients who have died of essential hypertension, we are immediately struck by the fact that the arterial changes in the choroid are far greater and earlier developed than in the retina. Several authors have observed this fact.

These changes of the choroidal arteries are of proliferative or degenerative nature and show widely differing pictures. In some, the changes in the intima are most pronounced, in others those of the media or the adventitia. In the intima considerable proliferation, which can be so advanced that the lumen is entirely blocked, is often observed. The elastic fibres have very little part in this proliferation. The thickened intima is often — but not always — inclined to fatty changes and, in exceptional cases, even to calcification. Lipidic macrophages are not uncommonly seen in the fatty deposits. The media principally shows degenerative changes in the form of hyalinization, and the hyalinized tissue often stains like fibrin (Fig. 1). It

can also give positive staining for fat. Such severe changes can be observed without thickening of the intima. Changes in the adventitia appear in the form of an increase in the number of nuclei. This is especially apparent if the vessel shows an aneurysmal bulging, as is sometimes the case (Fig. 2). Such increases in the cell nuclei have been demonstrated earlier by Kernohan, Anderson and Keith in many organs, amongst others in the skeletal muscles.

Of all these changes, only a number are simultaneously found in the arteries of the retina. As a rule, the preparations stained for fat give the most positive results. A considerable fatty change in the retinal arteries is often found without any remarkable changes being observed with ordinary staining methods in paraffin-imbedded preparations. It is remarkable that this fatty change spreads into the very fine branches (Fig. 3). The elastic fibres do not, as a rule, show any proliferation, nor has the present writer ever seen any hyalinization with fibrinoid degeneration, as in the choroidal arteries. The adventitia, however, at times shows somewhat considerable increase in the number of nuclei, and this is of particular interest since it explains the Salus-Gunn phenomenon. The Salus phenomenon, which in Anglo-Saxon literature is known as »arteriovenous constriction« or »arteriovenous nicking« consists, as it is known, in the disappearance of a blood column in the vein at an arteriovenous crossing, before the former reaches the artery, and reappears a short distance on the other side of the artery. It was formerly assumed that this was due to pressure on the vein by the artery. It is nowadays evident that the explanation is to be found in a thickening of the adventitia common to the artery and the vein, which makes the vein invisible for a short or long stretch. If the stretch is long, the Salus phenomenon is present. If it is short, it is the Gunn phenomenon, in which the vein is invisible at the actual crossing in front of or behind the artery. I have had the opportunity of examining such a crossing and found a very considerable increase in the number of nuclei in the adventitia, precisely as around an aneurysm in the choroid (Fig. 4). Such pictures can also be seen at the crossing of arteries and veins in the cerebrum, if the arteries show arteriosclerotic changes of a similar kind. Thickening of the adventitia of the retinal vessels has been observed by several authors, and Wagener (1937) goes so far as to consider that this is the principal cause of the ophthalmoscopically observable changes in the vessels in essential hypertension. This is, in my opinion, undoubtedly incorrect.

It is, however, not only in the form of the Salus-Gunn phenomenon, but also in many other ways, that the proliferative and degenerative changes described above are shown in the ophthalmoscopic picture. The correlation is, however, very uncertain, since only few authors had the opportunity to make microscopical examinations of retinal arteries, which were under observation during life.

Under normal conditions, only the blood column, but not the walls of the vessel, are seen. According to Ballantyne, Michaelson and Heggie, a blood column can, due to a thickening of the intima, be narrowed without the transparency of the vessel being noticeably changed. The cells of the intima do not then show any degenerative changes. When fatty change occurs, the walls become visible as sheaths surrounding the narrow blood column and what is called »parallel sheathing« is then present. With considerable fatty change the vessel becomes opaque and the blood column disappears and we have so-called »pipe-stem sheathing«.

The reflex streak, which normally occupies one-fourth to one-third of the width of the column of blood, is altered when thickening of the walls occurs. It is considered that the normal vessel reflex is due chiefly to light reflected from the blood column and, to a lesser degree, from the wall. If the wall thickens, the blood column narrows, but the reflex increases in breadth and intensity, since the wall component gains increased significance. In extreme cases, we have pictures of arteries which have been likened to silver wire.

If the narrowing of the artery depends on a contraction only, the reflex diminishes, on the contrary, in breadth and intensity. Gans (1944) thus asserts that, by means of the appearance of the arterial reflex, it is possible to differentiate between a narrowing caused by contraction and one caused by thickening of the wall. Both kinds can occur in essential hypertension.

The sclerosed arteries, however, often show yet another ophthalmoscopic change which is difficult to explain. They can thus be straighter than is normal and can branch at sharp angles. This has been explained as a result of a shrinking of the newly-formed tissue in the vessel in a longitudinal direction.

Finally, it should be mentioned that arteriosclerosis in the retinal arteries can take the form of a dilatation of the vessel. The arteries then become more sinuous and wider than is normal. The blood stream is visible through the comparatively thin wall and gives rise to a metallic reflex. The artery then has the appearance of a copper wire.

The sclerotic changes in the retinal arteries are characterized, as are the corresponding arteries in the kidneys and the cerebrum, by the fact that the changes are localized only to certain sections of the vessel in an irregular way.

The results of the hypoxaemia which is brought about by the changes in the vessels described above, are observed by ophthalmoscopy of the retina in the form of haemorrhages as well as white spots, which are of two kinds. These are either »soft«, ill-defined, and like pieces of cotton-wool, and can be of considerable size, or they are smaller, »hard«, well-defined and often starshaped, grouped around the macula.

Microscopic examination of the retina reveals a number of changes,

but it is not clear how these are to be correlated with the ophthalmological picture. They are partly degenerative and partly exudative in character. The degenerative lesions are partly varicose swellings of the nerve fibres (Fig. 5) arranged in groups, by which the layer of nerve fibres becomes locally considerably thickened, and partly by the appearance of fat, particularly in the inter-nuclear layer. The fat is either in the form of small droplets, on the fibres of Müller, or as large, shapeless extracellular masses or inside large fat-macrophages (Fig. 6). The pigmented epithelium can also show fatty change. Hyaline masses can be observed in the outer nuclear layer. These stain like fibrin (Fig. 5). It is difficult to decide whether these hyaline bodies are due to fibrinoid degeneration of the tissue or a fibrinous exudate. Fibrin appears further in the inter-nuclear layer as threads arranged in basket-shaped aggregates. It is clear that in the latter case it is a fibrinous exudate. The formation of oedema can cause a separation of the tissue elements, particularly in the inter-nuclear layer. Small haemorrhages can occur in all layers (Fig. 5).

In general it is considered that the hard white spots consist of an accumulation of fat and hyaline masses. No investigations show that these changes so widely differing anatomically appear different on ophthalmoscopy. There are, furthermore, no statements that the changes in the nerve fibres should manifest themselves in any particular way, which could perhaps be expected.

The so-called cotton-wool spots must be caused by oedema and not by fibrinous exudation. Such quantities of fibrin that they could correspond to these spots are not to be found.

Summary.

The author describes the histological changes in the vessels of the choroid and the retina in essential hypertension as well as the secondary changes in the retina.

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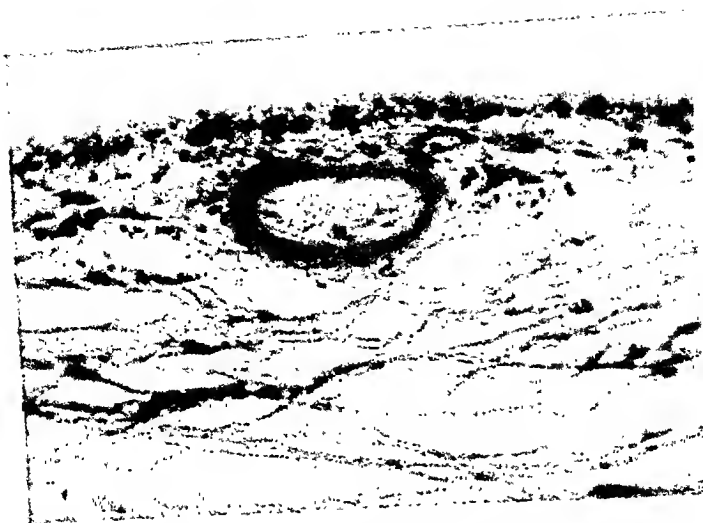


Fig. 1.

Hyalinization and fibrinoid degeneration of the walls of a choroid artery of a 39-year-old man, who died of uraemia due to malignant nephrosclerosis. B. P.: 230/150. The intima shows very little proliferation, and the elastic fibres are not hyperplastic. Severe lesions in the renal arterioles with fibrinoid degeneration, Mallory.



Fig. 2.

Artery in the choroid of a 46-year-old woman suffering from malignant nephrosclerosis, who died of cardiac insufficiency. B. P.: 280/150. Star-shaped deposits in the right macula. Papilloedema. Retinal haemorrhages. The wall of the artery shows hyaline and fibrinoid degeneration. In one place the wall shows an aneurysmal bulging and the adventitia at this site shows a considerable increase in nuclei. Haematoxylin-eosin. Staining for iron showed considerable quantities of iron pigment in the tissue around the vessel. In the retina, fatty change in the inter-nuclear layer and in the walls of a number of arteries (see Fig. 6). Haematoxylin-Eosin.



Fig. 3.

Fatty change in a fine arterial branch in the nerve fibre layer of the retina. Up to the left, the nerve fibres are swollen, 60-year-old woman, who died of cardiac insufficiency due to essential hypertension. Weight of the heart 720 g. Haematoxylin-Scharlach R.

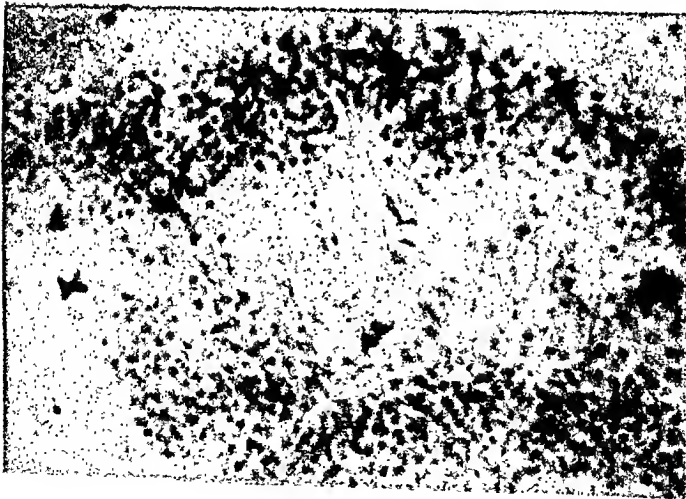


Fig. 4.

Arterio-venous crossing in the retina with considerable increase of cells in the common adventitia, in a 36-year-old woman, who died of severe nephrosclerosis with uraemia. B.P.: 245/175. Haematoxylin-eosin.

*Fig. 5.*

Picture of retina of a 45-year-old woman who died of uraemia owing to malignant nephrosclerosis, B. P.: 250/115. Up to the right is seen a thickening of the retina due to varicose swellings of the nerve fibres in the nerve fibre layer. Below, an increase in breadth of the inter-nuclear layer owing to oedema and haemorrhages (the red corpuscles are coloured red). To the left in the outer nuclear layer three hyaline bodies, which are also red, owing to the fact that they consist of fibrin. Mallory.

*Fig. 6.*

Fat in the inter-nuclear layer partly in the form of small drops on the fibres of Müller or within fat macrophages. For the clinical history see Fig. 2. Haematoxylin-Scharlach R.

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DISCUSSION

FREDRIK BERG: Professor Bergstrand mentioned that the vasoreflex is assumed to arise at the border surface between the vascular wall and the blood plasma. I will have to admit that I have never felt convinced about the correctness of this view. If a reflex is to arise it requires both a smooth border surface between two media and also a not all too small difference in the index of refraction for the two media. This requirement is met, above all, by the elastica of the vascular wall. Also certain ophthalmological observations indicate that the elastica is the site of the origin of the vasoreflex.

In the continued investigations on the correlation between ophthalmoscopic and histological changes in retinal vessels it was desirable if the attention focussed on the question about the anatomical foundation for the vasoreflex.

I. CHRONIC GRANULOMAS IN THE ALIMENTARY TRACT CAUSED BY MINUTE MINERAL PARTICLES

II. »BOECK'S DISEASE« AND OCCURRENCE OF MINUTE MINERAL PARTICLES

(Preliminary report).

By *Olav Refvem*.

I.

A series of conditions in the alimentary tract, such as gastric or intestinal cellulitis, regional enteritis, anal abscess, or anal fistula, have been regarded as foreign body reactions, isolated, or combined with a bacterial infection. Some investigators even hold that a large percentage of the cases diagnosed as ileocaecal tuberculosis, especially the hypertrophic varities, actually are foreign body granulomas. The sluggish passage in the distal part of the ileum and in the ampulla of the rectum should favor implantation in the intestinal wall and thus explain the prevailing localization in these regions. The presumption of a foreign body etiology in these cases is supported by few observations and mainly by theoretical deduction from this sparse material. Better methods for direct demonstration of foreign bodies in histological preparations will greatly improve our diagnostic results.

For this purpose the polarization microscope has been used systematically in a series of cases. In routine studies the polarization microscope has mainly been used to detect cholesterol crystals. This is probably due to the fact that the possibility of mineral granulomas has previously not been sufficiently considered, even where the type of inclusion bodies in foreign body giant cells has been widely discussed (Erdmann and Burt (1933)). Henschen (1924), however, used the polarization microscope to demonstrate foreign bodies in anal fistulas. He did not find any mineral particles, but found plant particles, oil droplets, mucous material, hairs, threads, etc. German (1943) and

others have demonstrated talcum particles as the cause of postoperative peritoneal granulomas and adhesions. *Gardner* (1937), in animal experiments, has shown that injections of silica in varying amounts and varying size of the particles may cause pictures resembling the different histological manifestations of tuberculosis, including formation of Langhans' cells and caseous necrosis. It has been observed repeatedly that long continued inhalation of quartz dust may cause pseudotuberculosis, not in the lung only, but also in other organs. *Elstad & Stenvik* (1946) found the same for olivine dust.

Professor *Kreyberg* has placed the archives of his private histological diagnostic service as well as the material of the University Institute of Pathology during the years 1935 to 1946 at my disposal. Because of service with the Armed Forces, Dr. *Kreyberg* has not been personally responsible for the diagnosis from 1940—45. The material selected for the present investigations consists of 209 specimens from the alimentary tract. Based upon the histological diagnoses, specimens from the following conditions were selected: Foreign body granuloma, granulation tissue, subacute and chronic non-specific inflammation, and chronic specific inflammation, as well as cases regarded as definite tuberculosis, the latter mainly from the ileocecal and ano-rectal regions. Relatively few cases of non-specific inflammation of the appendix were included. *Ulcus simplex ventriculi et duodeni* were entirely excluded.

Most of the sections examined were the original slides, stained after Masson's hematein-erythrosin-safranine method. In a few cases new sections were cut, if the original section was not satisfactory. The tissue material was not cut in series. Only single sections were examined. The number of foreign body granulomas discovered in the material, therefore, represents a minimum.

Where the foreign bodies are few and very small, the differentiation from smudge may be very difficult, and in pus practically impossible. To be accepted as a causative agent, the particles must be situated exactly in the same plane as that of the tissue, and be electively localized to, and naturally embedded in the pathological tissue. The cutting process may, however, cause solid particles to be displaced from their original site. Foreign bodies embedded in, or surrounded by giant cells, or within a cluster of leucocytes surrounded by fibrous tissue, are characteristic features. The main problem is to decide if the presence of the foreign bodies can explain the whole or part of the clinical picture.

Amanuensis of the mineralo-geological department of the Museum of Bergen, *Kvale*, Ph. D., has identified the substances as far as possible on the basis of their refractive properties. Certain errors in this type of diagnosis are evidently possible.

*Results.**Table I.*

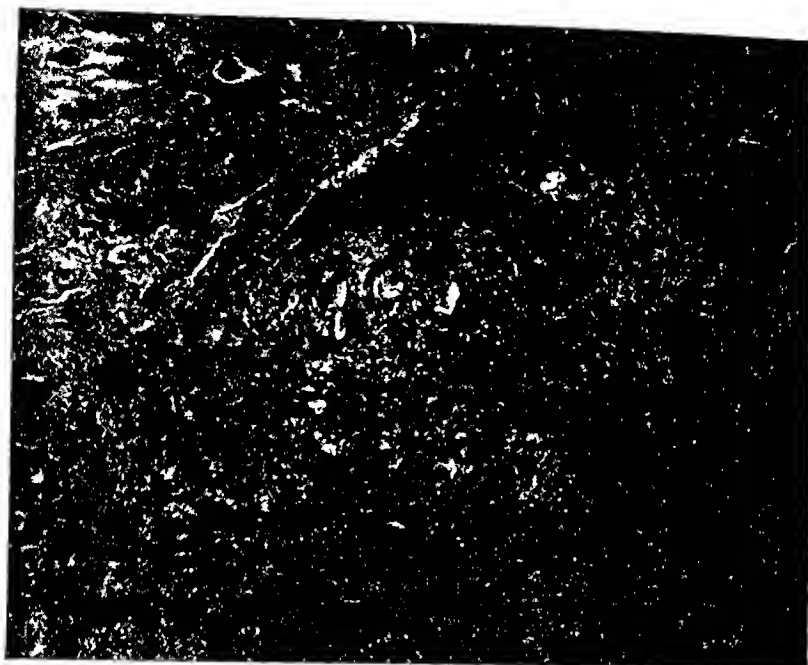
Nature of inflammatory condition examined	Number of cases examined	Cases with foreign bodies visible in polarized light:				
		certain			probable	
		Number	Percentage of cases examined	Possibility of foreign bodies previously mentioned	Number	Percentage of cases examined
For. body inflam.	16	11	69	11	3	19
Granulation tissue, subac. and chron. non spec. inflam.	82	30	37	9	11	13
Chron. spec. inflam. including the.	111	13*)	12	1	4	4
Total	209	53	25	21	18	9

*) Of these cases 4 presented a coincidence of tuberculosis and foreign body inflammation.

Table I shows that foreign bodies were found with certainty in 53 out of a total of 209 cases examined. Where foreign body reaction had previously been diagnosed, i. e., in 16 cases, 11 cases gave a positive reaction to polarized light and 3 of the remaining cases gave an uncertain reaction. In 82 cases, diagnosed as granulation tissue, subacute or chronic inflammation, minute foreign bodies visible with the polarization microscope were found in 30 cases, i. e., in at least every third case. In the series diagnosed as chronic specific inflammation, including tuberculosis, foreign bodies, as described, were found in 13 out of 111 specimens examined; 4 of these 13 cases, however, represented a coincidence of tuberculosis and foreign body inflammation.

Table II gives further details as to the occurrence of foreign bodies. As will be seen from table I and table II, the possibility of a foreign body reaction had in some instances been stressed or discussed in the original report of the case.

Table III shows the regional distribution of the cases. One will find, as might be expected, the highest incidence of foreign bodies in the ano-rectal region. In 34 per cent of all the ano-rectal specimens examined, foreign bodies were found, and talcum represented 75 % of all the foreign bodies in this region. This is mainly due to the high incidence (40 per cent) of foreign bodies in abscesses and fistulas. Foreign bodies may also be found in polyps, infiltrates, and ulcerations in the ano-rectal region. Contrary to expectation, only 2 out of 23 cases diagnosed as »tuberculosis possible« were foreign body granulomas, and of these two, one involved a fistula. In the rest of

*Fig. 1.*

Fistula ani. Histological diagnosis: »Chronic inflammation. Probably foreign body reaction«. In polarized light: Mineral granuloma, probably caused by talcum. $\times 100$.

*Fig. 2.*

A perforating tumor, the size of a tomato, in the jejunum, regional enteritis. Histological diagnosis: »Tuberculosis«. In polarized light: Mineral granuloma. Substance not identified. $\times 100$.

Table II.

Original histological diagnosis	Number of cases examined	Cases with foreign bodies visible in polarized light				
		certain			probable	
		Number	Percentage of cases examined	Possibility of foreign bodies previously mentioned	Number	Percentage of cases examined
For. body inflam. certain	16	11	69	11	3	19
» probable ..	6	2		2	0	
» possible ..	2	1		1	0	
	24	14	58	14	3	19
Tuberculosis certain	62	4	6	0	1	
» probable	20	0	0	0		
» possible	20	5	17	1	2	
	111*)	9*)	8	1	5	
Boeck's sarcoid?	3	2		0		
Lues?	9	1		0		
Lymphogranuloma vener.? ..	3	0		0		

*) In this number no case presenting both tuberculosis and foreign body inflammation is included.

the alimentary tract the average foreign body incidence was 20 per cent, of which nearly one half caused by talcum. One has not been able to confirm the suspicion that cases diagnosed as ileocecal tuberculosis to a large extent might actually have been foreign body granulomas. This was found in 2 out of 34 cases only. The incidence among the abdominal fistulas (4 out of 9) and peritoneal adhesions (6 out of 8, of which one with a coincident peritoneal tuberculosis) is remarkable. In one case an orange-sized tumor of the colon ascendens (regional enteritis) was due to large masses of cotton. A similar finding in the jejunum is demonstrated in fig. 2.

As to the nature of the foreign bodies (table III), the incidence of talcum granulomas is important, and it is in no way related to the chance of peroral ingestion of this substance. About two thirds of the »talcum fistulas« in the anal region occurred in men. Thus it cannot easily be regarded as to be connected with the use of facial or dusting powder. Talcum deposited at the rectal wall as a result of a digital examination is a far more reasonable explanation. The talcum undoubtedly is of importance for initiating or maintaining, an inflammatory condition. The demonstration of mineral particles in two cases of abdominal fistula support this view. Both cases occurred after an appendectomy. The talcum found in the peritoneal adhesions undoubtedly originates from the rubber gloves. Many surgeons do not even care to wash their gloves before the operation, and, furthermore,

Table III.

The regional distribution of foreign body granulomas of the alimentary tract caused by material visible in polarized light. Nature of the foreign bodies (certain findings) as identified by their refractive properties.

	Number of cases examined	Cases with for. bodies visible in polarized light							Tbc. (?)	For. body visible	For. body inflam. (?)	For. body visible
		Probable	Certain	Talcum	Blotite	Calcureous spar	Cotton	Not identified				
Abscess, fistula ..	52	4	21 40 %	16			1	4	17	1	8	4(5?)
Polypus	18	1	5	4			1		2	0	1	1
Ulcus, infiltr. etc. ..	12	2	2	1				1	4	1	0	0
Rectum + anus ..	82	7	28 34 %	21 26 %			2	5	23	2	9	5(6?)
Tbc., ileoc. region ..	34	0	2 6 %	1				1				
Large intestine	6	0	1				1					
Small intestine	13	2	1					1				
Stomach	6	0	0									
Abdominal fistula .	9	1	4	1	1			2				
Peritoneal adhesion	8	0	6	3 (4?)			3 (2?)		3	1		
Peritoneum	13	0	2	2				11	0			
Esophagus	2	0	1					1				
Oral mucosa, throat	13	2	3	1	1	1						
Other cases	23	6	5	3	1	1						
	127	11	25 20 %	11	3	2	4	5				
Digestive tract	209	18	53 25 %	32 15 %	3	2	6	10				
Total percentage ..				60	6	4	11	19				

not all the talcum is removed from the surface of the gloves even after several washings. In addition to the talcum from the surface of the gloves talcum may leak out from the inside, through rips in the gloves, during the operation, as shown by *Weed and Groves* (1942). Nor is a cloud of talcum dust surrounding the operating table an unusual occurrence. The cotton adhesions (2—3 cases) are also probably due to surgical dressings. Talcum and cotton present rather similar refractive properties, and one may sometimes be in doubt as to the actual type of foreign body. — A comprehensive literary review concerning the talcum problem, is given by *Seelig* (1943).

In a control material consisting of 50 cases of lymphogranulomatosis (Hodgkin's disease) foreign bodies could not be demonstrated.

II.

Histological sections from about 100 cases diagnosed as Bock's disease were examined in polarized light, each case represented by biopsy material from one or several of the lesions. In the course of this examination some interesting observations were made:



Fig. 3.

Postoperative intestinal adhesion. Histologically: »Chronic inflammation with foreign body reaction«. Talcum granuloma. $\times 100$.

1. In a male, aged 77, an ulcerating tumor, the size of a cherry, was found on the lower lip. The ulcer had lasted for half a year and was clinically regarded as a carcinoma. Four plum-sized submandibular lymph nodes of a firm consistency were removed. The histological diagnosis was »Boeck's sarcoid? Chronic benign tuberculosis?« In the polarizing microscope doubly refractile particles, recognized as calcareous spar, could be seen in the middle of some of the nodules, partly embedded in giant cells. Further clinical data from this patient are unfortunately missing.

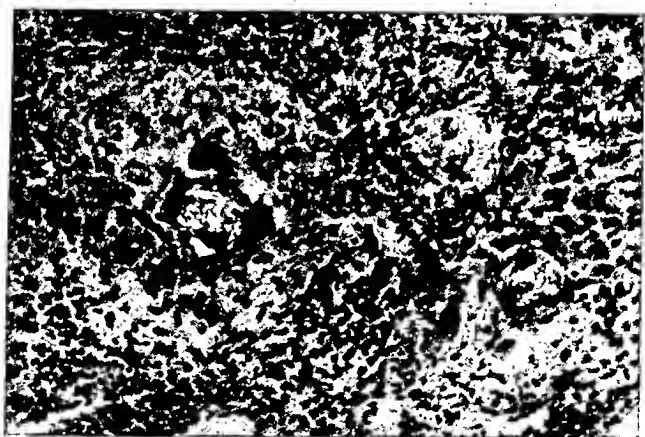
2. In a woman, aged 68, the diagnosis Boeck's sarcoid was made, based upon typical changes in the lungs with bilateral adenitis of the hilus, signs of previous iridocyclitis, foci of degeneration in the eyeground, a persistent negative Mantoux's test (with human and with chicken tuberculin 1 mg), and, finally, typical changes in a lymph node of the neck, the latter diagnosed as »Tuberculosis? Boeck's sarcoid?« In the description it is also remarked that the giant cells are »extremely large« and contain a blue-stained deposit, a feature some investigators consider a histological characteristic of Boeck's disease. In polarized light glistening particles are seen, partly in, partly close to the giant cells, some particles in direct contact with the blue-stained deposit previously mentioned.

3. A farmer, age 55, suffered from a disease diagnosed as Boeck's disease with multiple localizations (skin, lymph nodes, lungs, parotid glands, epididymides, eyes, metacarpal and finger bones) and of several years duration. The skin nodules, not quite typical resembled clinically a papulonecrotic tuberculide. Pirquet's reaction was positive, and he had a tuberculous fistula of the neck. T.B. could not be cultivated from the expectorate. Animal culture and inoculation from a large inguinal lymph node were also negative. Sections of this gland, skin plaques from arm, leg, and zygomatic region, presented the

usual histological picture of Boeck's sarcoid. The following year a tuberculous bursitis developed in the trochanteric region, and this was accompanied by a rapid and considerable regression of the exanthema and the swelling of the lymph nodes. A bone focus in the trochanter was surgically removed, and bacteriological and histological examination revealed tuberculosis. Control examination, 9 years later, shows that the exanthema has completely dis-



A



B

Fig. 4.

Section from inguinal lymph node in a patient with »Boeck's disease« and coincident tuberculosis. Histological diagnosis: »Boeck's sarcoid«. Detail photo taken in polarized light clearly shows mineral particles, identified as calcareous spar. Fig. A $\times 60$. Fig. B $\times 200$.

appeared. The pulmonary changes and especially the swelling of the lymph nodes are considerably reduced. After the operation a fistula has developed in the trochanteric region. Proteinuria and slightly impaired renal function still persist. There is no gross destruction of the kidney according to x-ray examination. Tubercle bacilli could not be cultivated from the urine or from the contents of a gastric lavage. Kveim's reaction has been positive for five weeks.

The especially interesting feature of this case is the finding of mineral particles (calcareous spar + quartz) in the previous biopsies: in the skin



Fig. 5.

Section from the nasal mucosa in a patient with Boeck's disease. Histological diagnosis: »Boeck's sarcoid«. Probably talcum granuloma. $\times 100$.

plaques from the face, the arm, and probably also from that of the leg. Also the large inguinal lymph node contains considerable amounts of calcareous spar (no quartz) in and around giant cells, and, as in case 2, partly in direct contact with the blue-stained deposit (fig. 4). At the control examination 9 years after the original biopsies were made, a biopsy was taken from a conjunctival nodule, from the nasal mucosa, and from lymph nodes in the axilla, but there was no evidence of specific histological changes or mineral particles, the axillary lymph nodes having been converted into a fibrous tissue.

4. A man presented a typical case of Boeck's disease with multiple localizations. Biopsies were made from several of the lesions, and all of them were histologically diagnosed as *Boeck's disease*. By examination in polarized light a section from a nodule of the nasal mucosa was revealed as a foreign body granuloma, probably caused by talcum (fig. 5). In biopsy material from other lesions, however, no foreign body could be demonstrated.

5. Examination of excised papules of Kveim's reactions has in a few instances revealed a crystalline substance in the centre of the specific(?) histological lesions (fig. 6 and 7). These crystals probably were introduced with the antigen. Their origin is uncertain. They may have been in the tissue from which the antigen was prepared, but, more probably, they are artefacts from the grinding of the tissue.

6. In addition, a clinical observation concerning the same problem: Two workers in a factory of rubber articles had been much exposed to inhalation of chalk powder. They both presented roentgenological changes in the lungs resembling those seen in Boeck's disease. The Pirquet's and Mantoux's tests were negative in one of the patients. The other patient had



Fig. 6.

Skin section from papule developed after injection of antigen (Kveim's reaction). Histological diagnosis: »Chronic specific inflammation with necrosis». $\times 100$.



Fig. 7.

Same section (fig. 6) in polarized light. In the left lower corner a large crystalline particle in a giant cell, and in the nodules very small doubly refractile bodies can be seen. In the centre a cotton thread (in the canada balsam). $\times 100$.

in 1936 a bacteriologically and histologically verified tuberculosis of the ilio-sacral joint with positive Pirquet's reaction, and miliary infiltrations of the lungs. The latter patient now reacts negative to Pirquet's and Mantoux's tests. Tubercle bacilli have not been demonstrated in the expectorate of these patients. On account of the localization of the disease, biopsy could not be made. The cases have been clinically registered as Boeck's disease, pulmonary form.*)

Discussion.

In 4 patients presenting lesions with the histological picture of Boeck's sarcoid, crystalline material has been found in the nodules. At least 3 of these 4 patients presented also the clinical picture of Boeck's disease (cases 2, 3, 4). The fourth (case 1) presented a histological picture very similar to the Boeck lesions, but the clinical data of this patient are very inadequate and do not permit a clinical diagnosis.

In one of the definite Boeck cases (case 4) mineral particles were found in one lesion only, viz. in the nasal mucosa. In the two other cases with a clinical and histological picture of Boeck's disease, the presence of foreign bodies cannot easily be dismissed as accidental. It is possible that in these cases a more or less generalized systemic disease caused by mineral particles has developed. This disease may be an independent entity presenting a more or less complete picture of Boeck's disease and constituting a certain number of the cases usually diagnosed as Boeck's disease. The experimental findings of *Gardner* (1937) and *Elstad & Stenvik* (1946) add evidence to the explanations given in this presentation. If this theory is accepted, it will be possible to explain the symptoms presented by the two workers in the rubber factory, mentioned in paragraph 6, above, as caused by inhalation of chalk-dust, which is chemically the same substance as calcareous spar, only amorphous. In the other cases presented above information about exposure to mineral dust was available in case 3 only, the patient being a landworker partly employed in a peat drying factory and so exposed to large amounts of peat dust.

An additional case (skin infiltration) would have been described as Boeck's disease, had it not been for the information of a spurt of stone particles against the site of the lesion. The history led to the finding of crystals in giant cells, and thus the diagnosis of foreign body granuloma.

That oils, paraffin, and necrotic body fat can produce granulomas very similar to the Boeck lesions has been known for many years.

The following question arises: Is it possible to explain a certain

*) The cases presented under paragraph 2., 3., and 6., refer to patients from the medical departement B, of the University Hospital. The author is very much indebted to the chief, professor H. A. Salvesen, M.D., for his permission to use the material.

number of cases of »Boeck's disease« as a more or less generalized foreign body reactions, in cases limited to individuals with a special mode of reaction? The etiology may in such instances be regarded as polymorphous, or connected with certain specific substances. On the basis of the calcareous spar particles found in the lesions, it may be of interest to consider the chance of exposure to this substance. It is a well-known fact that people living in the country are preferably attacked by Boeck's disease, especially farmers, gardeners, etc. *Låg*, at the Norwegian High School of Agriculture, points out that the soil in cambro-siluric regions may contain particles of calcareous spar, with diameters of only a few microns, which may be whirled up as dust and thus be inhaled. Furthermore, lime fertilizers contain a mixture of quick-lime and lime-stone, i. e., calcic carbonate. This substance may be inhaled and thus be deposited in the air passages and in the lungs, or other places, and cause localized forms of Boeck's sarcoid. Through lymphatic and hematogenous spread the particles may produce a more generalized disease. That mineral particles are not more constantly found in the Boeck lesions may be due to the circumstance that they are responsible only in a limited number of cases. But the explanation may also be that extremely small quantities, which may easily be overlooked in microscopic examination, are sufficient to cause changes in a sensitized (?) or even normal, organism. Or the material may successively be removed, dissolved, or converted into amorphous, non-injurious compounds, cfr. the amorphous inclusion bodies in giant cells (calcareous deposits?). This last feature may explain the involution of Boeck manifestations occurring in some instances. Regression coinciding with an active tuberculous phase have also been observed. Perhaps this may be caused by a change of the surrounding tissue milieu, rendering it able to neutralize or convert the irritant agent. In case 3, considerable lesions regressed in the course of one year and a biopsy 8 years later did not reveal characteristic histological changes, nor mineral particles.

It is possible that the so-called atypical and slightly virulent species of tubercle bacilli, which are proposed to be responsible for some cases of Boeck's disease, and which cannot be cultivated, actually are

Summary.

I.

Material representing various chronic inflammatory conditions in the alimentary tract was examined in polarized light in order to find out if the causative agent might, in some cases, be foreign bodies, especially minute mineral particles. In at least 53 out of a total of 209 cases examined, i. e., 25 per cent, such foreign bodies were found. The incidence was highest in the group consisting of granulation tis-

sue and subacute and chronic non-specific inflammation, viz. 37 per cent. Also among cases originally registered as chronic specific inflammation including tuberculosis, however, the number of foreign body granulomas was significant (12 per cent). Four cases, though, represented both tuberculosis and foreign body inflammation. Out of 62 cases with a »definite diagnosis of tuberculosis«, 4 (i. e., 6 per cent) actually were foreign body granulomas.

As might be expected, foreign bodies were especially found in specimens from the ano-rectal region, viz. in 34 per cent. Contrary to expectation, foreign bodies were demonstrated only in a few sections from the ano-rectal and the ileocecal regions diagnosed as tuberculosis. Foreign bodies, mainly talcum and cotton particles, were found in most of the cases of abdominal fistula and peritoneal adhesions. Especially interesting, in 2 cases of regional enteritis, was the occurrence of mineral particles, and cotton, respectively. This will support the theory of a foreign body etiology in such conditions.

The particles were identified by optical analysis. Talcum was the agent responsible in 60 per cent of all the foreign body granulomas found in the alimentary tract. This was mainly due to the high incidence of talcum granulomas in the ano-rectal region, a fact which is most reasonably explained by the digital examination with powdered rubber gloves. — Other foreign bodies were identified as biotite, calcareous spar, and cotton, whereas the nature of the substance in a number of instances remains uncertain.

II.

In 4 patients crystalline material was found in nodules presenting the histological picture of Boeck's disease. At least 3 of these patients also presented characteristic clinical features of this disease. Two of these cases most probably represented a hitherto unrecognized entity, which may constitute a certain number of the cases usually diagnosed as Boeck's disease. A report is made of 2 cases of »Boeck's disease«, pulmonary form, possibly caused by inhalation of chalk dust.

Based upon the observations of other investigators and those of the author, the possibility that foreign bodies more frequently cause »Boeck's disease« is discussed, and the possibility of a gradual breaking down or even complete disappearance of certain foreign bodies in the tissue, especially varieties of calcic carbonate, is considered.

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DISCUSSION

HEERUP, L.: There can be no doubt that in earlier days the diagnosis of tuberculosis was made all too often and erroneously — as, for instance, in cases of analfistula and chronic otitis, among which, in earlier days, 20 % were claimed to be tuberculous. In the past ten years I have examined all scrapings from chronic ear lesions in the Frederiksberg Hospital, and do not remember ever having seen any case of tuberculosis in the whole lot.

To me it seems highly interesting that several cases of the Boeck-Schaumann disease have proved possibly to be foreign-body granulomas. I will admit that it is merely a matter of belief, but I do not think that the Boeck-Schaumann disease really is any disease *per se*, but most frequently only a particular tuberculous reaction, and now we see that several cases of this kind possibly may have an entirely different pathogenesis.

The exception of the histological features encountered in the affection of the lymph nodes previously designated as Ziegler's large-cell hyperplasia, I should not venture myself to make the diagnosis Boeck-Schaumann's disease but merely designate the specimen as: tuberculoid changes, or: tuberculosis?, or: Boeck-Schaumann's disease?

REFVEM, O.: With regard to the relation between the bluish particles observed in the giant cells and adjacent crystalline particles of calcium carbonate, I have mentioned merely as a possibility that the latter might be rendered harmless by transfer to the non-irritating amorphous lime sediment, which consists chiefly of 9 parts calcium phosphate and 1 part calcium carbonate. In this connection I wish to recall the wellknown fact that the product of Ca and P cannot exceed a certain value without giving precipitation.

Dr. Heerup thinks that Boeck's sarcoid is almost exclusively a tuberculous manifestation. In this connection I wish to remind of cases of tuberculosis and Boeck's sarcoid, in which calcium carbonate was

found in several of the lesions. This seems to indicate that even in a definitely tuberculous patient some lesions bearing the Boeck pattern may still have been caused, not by tubercle bacilli, but by crystalline particles, such as calcium carbonate. I frankly admit, however, that arguments for accepting this substance as a causative agent, so far, are based merely upon analogism. Animal experiments have been started in order further to elucidate this problem.

THE PATHOLOGICOHISTOLOGIC LIVER PICTURE IN CONTAGIOUS HEPATITIS IN DOGS

By S. Rubarth.

Contagious hepatitis in dogs (*Hepatitis contagiosa canis*), which has been found to be caused by a virus (Rubarth)¹), is a widespread disease in Sweden. Most dogs seem to pass through it without, or with merely minor, clinical symptoms, whereas only a relatively small number of cases have a fatal issue. By means of the complement fixation reaction, it has been shown that about 75 per cent. of the dogs in the Stockholm district have antibodies against this virus.

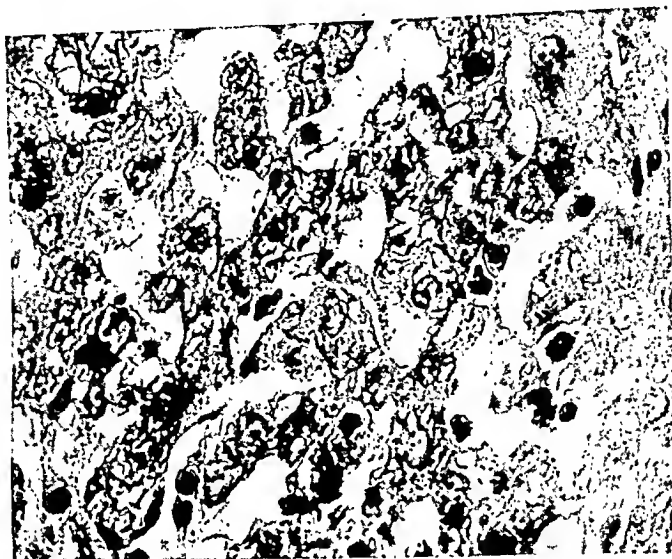
The pathologico-anatomical picture is marked by a diapedetic passage of liquor and of erythrocytes, with inflammatory changes especially in the liver, lymph-glands, spleen and often also the brain.

The histologic liver picture is characterized by hyperemia and edema or hemorrhage into the Disse's spaces. This diapedesis, which begins centrolobularly, is accompanied by nuclear inclusions and other degenerative changes in the endothelial cells. The regressive changes in the liver-cells likewise begin centrolobularly and may show marked variations, from mild lesions to complete necrosis; in the latter case the cell remnants lie as in a hemorrhagic pool of blood. The «eosinophil degeneration» of the liver-cells found by Axenfeld and Brass²) in *Icterus catarrhalis* in man occurs in these cases too, as well as nuclear inclusions in greatly varying number. A migration of nucleated blood-cells sets in rather soon. A by no means unusual finding is a phagocytosis of red blood-corpuscles in the liver-cells, and sometimes phagocytosed leukocytes may also be observed. Within the regressive centrolobular areas, fibrinous precipitations often occur both in the sinusoids and in the Disse's spaces; in such cases minor fibrinous

1) Rubarth, S.: An acute virus disease with liver lesion in dogs (*Hepatitis contagiosa canis*). Acta Pathologica et Microbiologica Scandinavica. Supplementum LXIX 1947.

2) Axenfeld, H. und K. Brass: Klinische und biopsische Untersuchungen über den sogenannten Icterus catarrhalis. Frankfurt. Ztschr. f. Path. 57, 147.

threads are rather frequently found in vacuoles in the cytoplasm of the liver-cells (fig.), a finding which I have explained as a manifestation of a phagocytosis, similar to the above-mentioned erythro- and leukophagy.



Liver from dog with short fibrin threads phagocytosed in the cytoplasm of the liver-cells. Nuclear inclusions in the liver- and endothelial cells. Carnoy fixation, Weigert's fibrin stain and alumi carmine. 1000 X.

The nuclear inclusions which occur in the liver- and endothelial cells, and which are of Cowdry's type A, usually have an eosinophil character, varying, however, according to the fixing of the material and the staining method employed. These inclusions are not only an indication of the action of the virus, but also a sign of nuclear damage that leads to the destruction of the cell. It is therefore not surprising that in the most acute and severe stage of the disease nuclear inclusions are found in abundance, whereas in the cases which run a slower course with a lethal issue a sharply delimited centrolobular necrosis, with extremely sparse inclusions or none, is observed. The violent diapedesis of liquor or erythrocytes that occurs in the liver is reflected also in the gall-bladder wall and in the abdominal cavity, where a copious amount of a serous fluid sometimes of hemorrhagic character, is often found.

NO DISCUSSION

ISLETS OF LANGERHANS IN FIBROCYSTIC DISEASE OF THE PANCREAS. A HITHERTO UNDESCRIBED ABNORMALITY?

By *Olav Torgersen.*

The condition commonly named fibrocystic disease of the pancreas has been described under a series of different names during the past 30—40 years. The characteristic pancreatic changes were probably first described by *Landsteiner* (1905) in the so-called meconium ileus. It soon became apparent, however, that similar pancreatic lesions could be found at autopsy in children dead under different clinical pictures. The present conception of the disease as a pathological entity is based on systematical work of later years *inter al.* by *Blackfan* and *Wolbach* (1933), *Dorothy Andersen* (1938), *Blackfan* and *May* (1938) and *Menten* and *Middleton* (1943). The classification now commonly adopted is essentially that of *Andersen* (1938) according to which three main groups are differentiated:

I. The most acute form, in which the patients generally die within the first or second week after birth. The clinical picture is the so-called meconium ileus. This form is the least common, occurring in about 10 per cent of the cases.

II. A more subacute form, in which the patients generally die within the first year of life. This is the most common form. The predominating symptoms are from the gastrointestinal tract (diarrhoea, vomiting) and from the respiratory tract (severe respiratory irritation terminating in bronchopneumonia).

III. A rather rare and more chronic form in which the patients may live more than one year. The symptoms of the initial stage are largely the same as those of the preceding group and the disease begins at an early date, but later there may appear symptoms of celiac disease. These patients, too, are prone to succumb from widespread suppurative pulmonary infection.

The most characteristic finding at autopsy in all types is the lesion of the pancreas. The excretory ducts are dilated and cystic, lined by a low cylindrical or cuboidal epithelium. The ducts contain an apparently massive, acidophile secretion which is often arranged

in concentric layers and gives a positive mucin reaction. The acini are more or less atrophic. The interlobar and the interacinar connective tissue is very markedly increased and there is a leucocytic infiltration of varying intensity. As an apparently sharp contrast to the severe lesion of the exocrine apparatus the islets of Langerhans are described as normal by almost all authors. This feature has even been used as an argument against the conception that the glucosuria found in certain cases of pancreatic cysts might be of pancreatic origin (*Lindan, Gruber* 1929). Contrary to other authors, *Baggenstoss* and *Kennedy* (1945) found slight but definite abnormalities in histologic structure of the islets in most of their cases. They found that the cells were loosely arranged in bands and in a few cases intercellular edema was present. An interesting statement was made many years ago by *Margrith Teuscher*: she wrote: »Die Langerhanschen Inseln sind noch sehr klein und stehen mit den Ausführungsgängen in direkter Verbindung«.

In the literature available to me, however, I have found no mention of the lesion or abnormality of the islets which will be described below. The lesion was found in the five cases of fibrocystic disease in which I have had opportunity to examine microscopic sections of the pancreas. Three of the cases have been described previously, from a clinical standpoint, by Dr. Alf Ödegård, and the cases will be more fully dealt with elsewhere. Here will be given a brief description of the cases, with special attention to the structure of the islets of Langerhans.

Case 1: A 4½ months old female child. Weight at birth 3090 g. Three weeks after birth the child began to vomit (partly projectile vomiting) and gained poorly in weight. Later the stools became loose and greenish. X-ray examination revealed an opacity of the left lung. *Autopsy* (O. 15/44): Fibrocystic disease of pancreas. Fatty metamorphosis of liver. Suppurative bronchitis and peribronchitis of left lung.

Case 2: A 4 month old female child. Weight at birth 4160 g. Gained poorly in weight. Three months after birth vomiting, fever and severe cough. Increasing cachexia. *Autopsy* (O. 340/44): Fibrocystic disease of pancreas. Fatty metamorphosis of liver. Catarrhal bronchitis. Calcium salt deposits in the kidneys.

Case 3: A 2 months old male child. Weight at birth 4000 g. Gained poorly in weight. 2 weeks after birth there appeared a bullous exanthema. The stools became loose and greenish. The last days before death some vomiting.

Autopsy (O. 340/45): Fibrocystic disease of pancreas. Slight fatty metamorphosis of liver. Bronchopneumonia.

Case 4: A 2 years old male child. Weight at birth 3000 g. 4—5 months after birth severe cough and febrile periods. Gained poorly in weight. One year after birth the stools became loose, and later they were found to contain rich amounts of fats. The blood sugar curve was flat. The child succumbed to repeated infections, terminating in an abscess of the right lung.

Autopsy (O. 269/46): Fibrocystic disease of pancreas. Fatty metamorphosis of liver. Abscess of right lung.

Case 5: A 7 months old male child. Gained poorly in weight since birth. Fatty stools and severe pulmonary infection.

Autopsy (Pp 1892/47): Fibrocystic disease of pancreas. Bronchopneumonia.

The changes in the exocrine apparatus of the pancreas corresponded fully to the descriptions given in other papers and will not be repeated here.

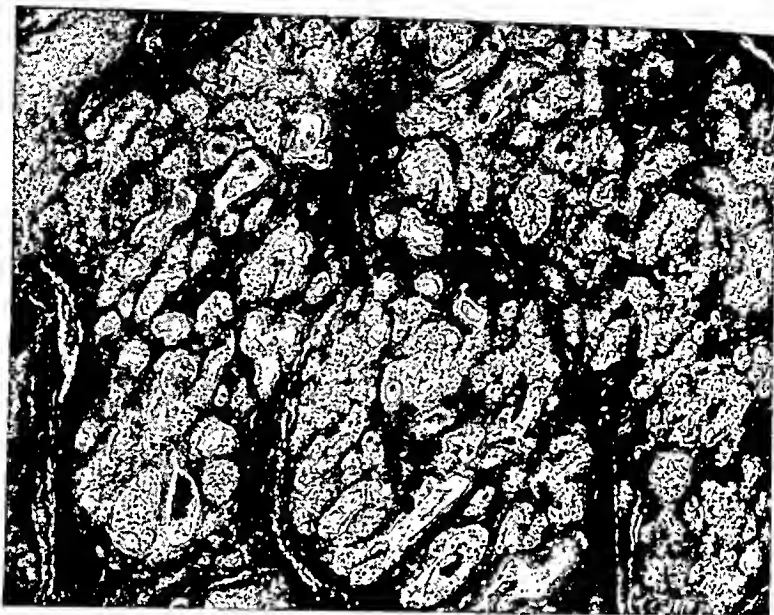


Fig. 1.

Low power view of pancreas in case 4, showing the typical picture of fibrocystic disease. Zenker-formol fix., Mallory-azan. $\times 60$.

The *islets of Langerhans* were present in fairly normal amount. Many islets appeared normal or slightly edematous. At closer examination many of the islets were found to contain one or more duct-like lumens, lined by an epithelium of somewhat varying structure. In the rarest but most impressive specimens the epithelium was of the tall columnar type with basally situated nuclei and a coarsely vacuolated cytoplasm which gave a positive mucin reaction (mucicarmin), the whole resembling closely small pancreatic excretory ducts (fig. 2). More often the ducts were less conspicuous, cleft-like and were lined by a low stratified epithelium. In these cases the ducts might be easily mistaken for blood vessels (fig. 3), except for their content which was amorphous and generally gave a positive mucin reaction. In still other instances the ducts were wider and their epithelium showed degenerative changes. The contents of these ducts were partially made up of necrotic material (fig. 4). A great many sections were cut to find out if the ducts within the islets had any connection with the exocrine apparatus of the pancreas. In several instances a »hilus« could be demonstrated in which the ducts seemed to leave the islet of Langer-

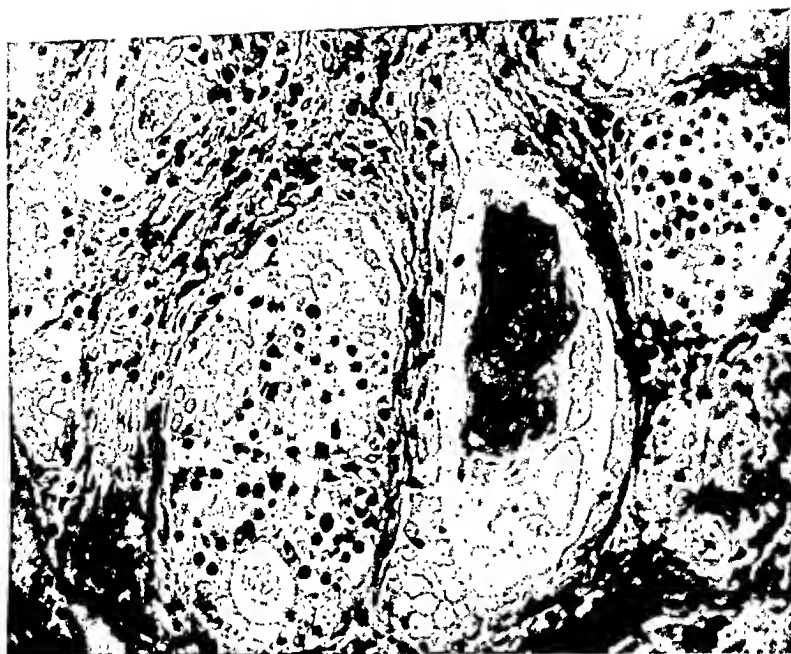


Fig. 2.

Islet of Langerhans containing three duct-like lumens, lined by a more or less tall columnar epithelium. Note the resemblance of these structures to the small excretory duct joining the larger, cystically dilated duct in the middle of the picture. Right: apparently normal islet. Detail of fig. 1, Zenker-formol fix., Mallory-azan. $\times 350$.

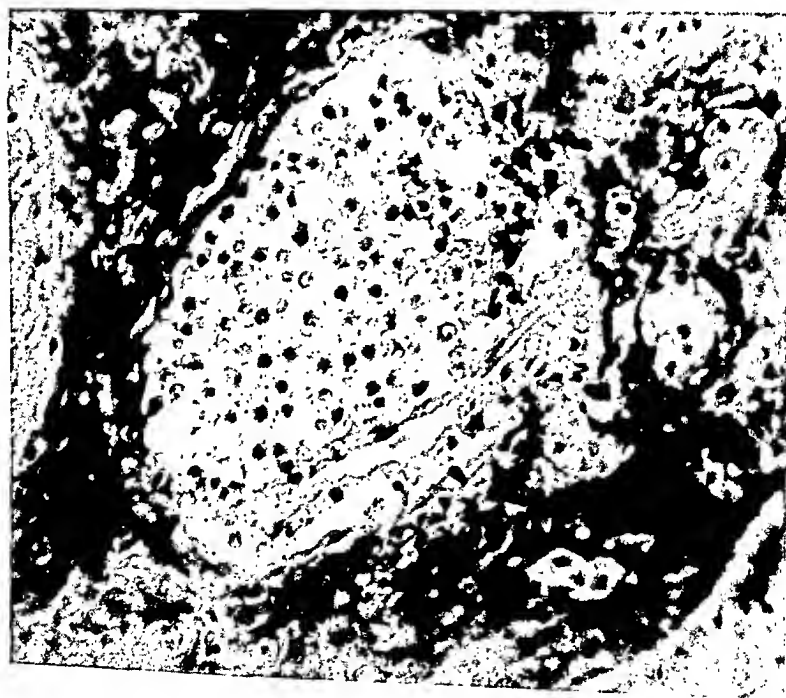


Fig. 3.

Islet containing a cleft-like duct simulating a blood vessel. Case 1. $\times 600$.

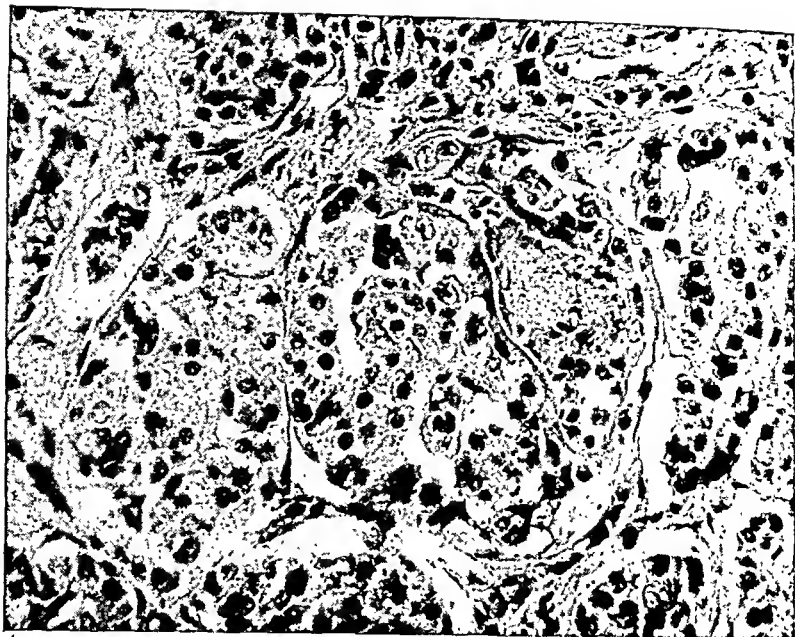


Fig. 4.

Islet containing a rather wide duct with flattened epithelium showing retrogressive changes. The contents are mucinous, partly mixed up with degenerating cells. Case 3. Formalin fix., Mucicarmin. $\times 400$.

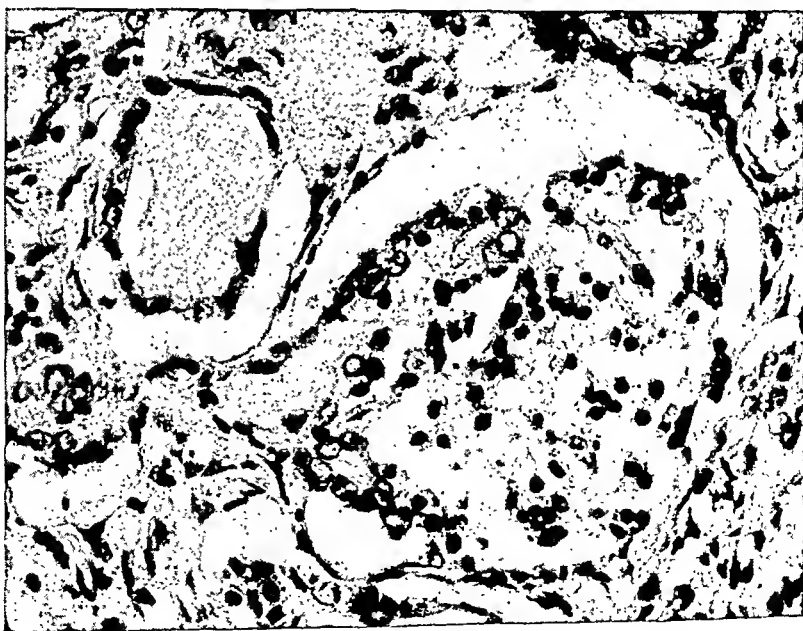


Fig. 5.

Islet resembling to some extent a glomerulus. Branching duct leaving the islet at the «hilus». Case 5. Formalin fix., Hematox.-eosin. $\times 400$.

islets apparently to join the nearest small excretory duct, the whole resembling to some extent a glomerulus (fig. 5).

In one case serial sections were made to see if there actually existed islets which were completely devoid of ducts. The sections showed that at least in one instance no duct could be found within the islet in question. In one case attempt was made to determine the amount of A and B cells within the islets. In this case no abnormalities of these cells could be detected.

To explain these features one might consider two main possibilities.

First, the ducts described might be considered as newly formed elements analogous to hyperplastic bile ducts (or liver cells) found

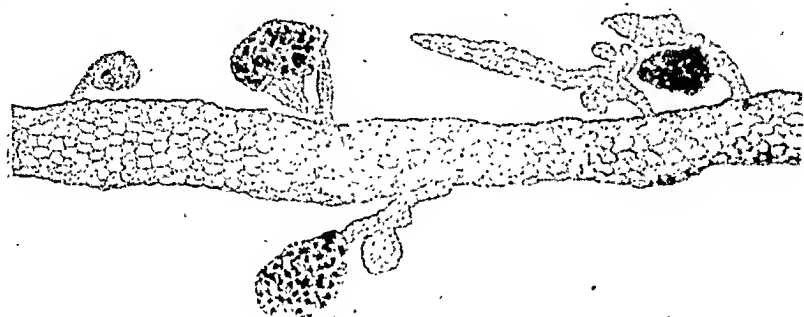


Fig. 6.

Connections between islets and excretory ducts in the Guinea pig.
After Bensley (1911).

in cirrhosis of the liver, i. e., a process probably secondary to degeneration and fibrosis. This possibility has probably to be abandoned. In fibrocystic disease, there is no appreciable hyperplasia of the small excretory ducts within the pancreatic connective tissue, and it would seem highly improbable that newly formed ducts should proliferate into the islets of Langerhans only. Furthermore, the ducts in question are well represented in case 3, i. e., in a child aged 2 months where the changes in the exocrine parts of the pancreas are rather small.

The second and far more probable possibility is that the ducts described may be related to, or identified with, the peculiar small tubules described at the beginning of this century, inter al. by *Laguesse* and which were beautifully demonstrated by *Bensley* (1911) in Guinea pigs (fig. 6) by means of vital staining. The epithelium of these tubules is of the irregularly low cuboidal type with occasional goblet cells and a few cells with true mucous granules. According to current textbooks of histology these structures represent a matrix of undifferentiated epithelium from which the regeneration of islet tissue may occur. They are not believed to carry any secretion. Although studied most extensively in Guinea pigs, the tubules are also said to be present in man, but systematical investigations on this subject seem to be lacking. The occurrence of such structures is easily explained when

one considers the genesis of the islets from the entodermal excretory ducts in fetal life.

The peculiar thing is that, in fibrocystic disease, at least, these tubules seem to persist in postfetal life, within otherwise fully developed islets of Langerhans. Further, the tubules may appear as well differentiated structures, and, finally, their epithelium may become the seat of retrogressive changes.

The significance of these investigations must be decided by future research.

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DISCUSSION

ASK-UPMARK: I should like to know whether the mothers of these children perhaps have had an attack of rubella during pregnancy. Was any instance of bronchiectasis observed in these cases?

O. TORGENSEN: To Professor Ask-Upmark I wish to say that there are no available data as to the occurrence of rubella during pregnancy in the cases here concerned. The case histories were taken at a time, however, when we were not so well orientated concerning these phenomena, so that hardly any significance may be attached to the records on this point. As to bronchiectasis, this morbid condition has been demonstrated in several cases reported in the literature, but not in any of our cases.

AN INVESTIGATION ON PREGNANCY IN DIABETIC ANIMALS

By Gösta Hultquist.

Pregnancy in a diabetic woman causes changes in the physical aspects of the disease, and the diabetes, in its turn, affects the pregnancy and the offspring. In general, the disease seems to grow worse, but a few cases have been described in which the diabetes of the mother became less severe during the later stages of pregnancy.

In order to study the conditions prevailing in diabetes pregnancy and the underlying pathologico-anatomic aspects, I have been experimenting for some years on rats. The experiments have been extremely difficult to carry out, but I have now assembled a material consisting of about 20 cases which are being studied from the histologic standpoint. The present paper is a preliminary report on the investigation.

In most instances, diabetes was produced in the animals by subtotal pancreatectomy and in a few cases by means of alloxan. The majority of the animals were operated on at varying times after the start of the pregnancy. Only in a few instances (in alloxan animals, among others) did pregnancy occur in animals already suffering from diabetes. The blood sugar (by Folin's method) and urine sugar (Benedict's reaction) were studied. Most of the animals were treated with insulin.

The clinical course of the diabetes in the mother rat varied widely in the different animals, in spite of the fact that the excision was done as far as possible to a uniform extent in all cases. In some animals the increase in the blood sugar was only slight, the values being around 0.15 g. per hundred cc. (the normal value for this breed of rat is around 0.08 g.), whereas in others figures around 0.4 g. were obtained (as in rat R 211, in fig. 1). In a couple of the animals becoming pregnant after they had had diabetes for some time the blood sugar values rose during the pregnancy, and there was a strong tendency towards fluctuation. A value as high as 1.45 g. per hundred cc. was obtained on one occasion from a pregnant alloxan rat. A strong decrease in the blood sugar generally occurs immediately before

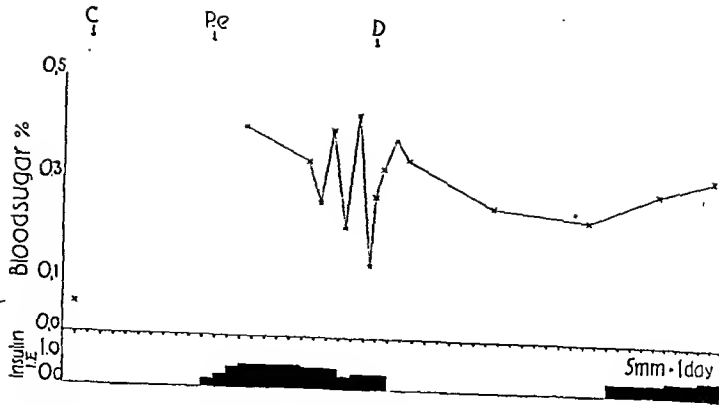


Fig. 1.

Blood sugar curve for pregnant rat (R 211) with diabetes following pancreatectomy. C = conception. P.e. = pancreatectomy. D = delivery.

delivery, as may be seen from figure 2, which shows the values from a number of experiments five days before and after parturition. The curve was obtained by adding the figures and calculating the mean values for shorter periods. The curve runs fairly level except during the 12 hours immediately prior to delivery, when a noticeable drop takes place. The decrease is relative and hypoglycemic values were

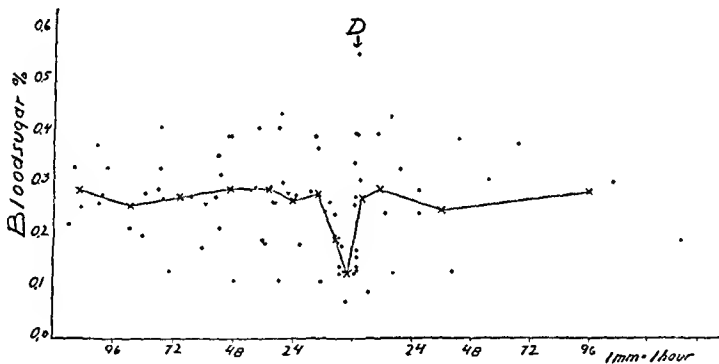


Fig. 2.

Blood sugar values obtained in experiments 5 days before and after delivery, and curve showing the mean values.

not observed. As a rule, there is an increase in the blood sugar after or during parturition. In these animal experiments, there was no improvement in the diabetes of the mother rat during the later stages of pregnancy such as is observed occasionally in human beings. Increased sensitivity to insulin and a tendency towards a decrease in the sugar in the urine is, however, often observed even before the blood sugar decrease occurring prior to parturition, and if insulin is being administered in these cases the amounts must be reduced in consequence.

How are we to explain the strong decrease in the blood sugar during the hours before parturition? If the decrease were due to compensatory hyperplasia of the islets in the fetus with the transference of the insulin to the mother rat, then it seems strange that such a state should arise so suddenly, just before parturition. A decrease in the blood sugar level before parturition is observed in normal subjects also, but the drop is not so marked as it is in animals with diabetes. One possible explanation of the phenomenon may be that the permeability of the placenta increases immediately prior to parturition, thus making it possible for insulin to pass from the fetus to the mother. According to *Marshall, Harris* and others an increase in the placental permeability takes place on delivery or in the later stages of pregnancy. Degenerative changes in the placenta are demonstrable in normal subjects at the termination of a pregnancy (*Huggett*).

The experiments have shown that treatment with insulin is definitely of value for achieving full-term pregnancy. In a long series of experiments without insulin administration abortion occurred soon after the pancreatectomy (this observation tallies with the experiences of *Long*, as reported by *Miller*), and full-term pregnancy was achieved in only one rat. When insulin was given, the results were more favourable. The size of the dose would seem to be of no particular significance. Success was obtained both with large doses and with doses so small as to exercise no noticeable effect on the blood sugar level. A good illustration of the effect of insulin treatment is provided by a rat with diabetes produced by alloxan. The rat was kept in a cage together with potent male rats for a little over one month without becoming pregnant. After two weeks' treatment with small doses of insulin pregnancy occurred and continued to full term.

Increased weight at birth in the infants of diabetic women has often been reported, and there has been much speculation as to the cause.

The birth weights of the young rats in my experiments are shown in table 1. For the most part the weights of the offspring of the diabetic rats fall within the standard deviation of the control series. On the other hand, in 3 rats (R 195, R 211, R 212) there is a clear, and to some extent a strong increase in the weights of the offspring, the values going as high as 7.4 g. This is an increase by more than 50 per cent over the normal mean value for the breed of rat used in my experiments. These weights fall outside the standard deviation of the normal value, and are thus statistically significant. In the case of the giant infants it is not only a question of an increased accumulation of fat but also, more particularly, of macrosomia with splanchnomegalia.

In the few animal experiments on diabetes pregnancy reported in the literature it has not been possible to reproduce the conditions observed in human beings, namely, large fetuses in some diabetic

Table 1.
Weight of the rat infants.

	Rat no.	Number of offspring	Weight			Comments
			Min.	Max.	Average	
Diabetes	208	11	1.85	2.929	2.221	Parturition > 2 days too early
pancreatectomy	228	3	2.950	3.348	3.213	Parturition at full term. The other rat had transient icterus.
»	198	6	3.208	4.213	3.476	Parturition at full term. Diabetes only 5 days.
»	210	11	2.947	4.014	3.489	Exact time of conception known.
»	214	5(10*)	3.309	4.525	4.073	Parturition at full term.
»	152 _{II}	11	3.250	4.779	4.274	Exact time of conception known. No insulin.
»	202	7(8)	3.903	4.688	4.331	Exact time of conception known.
»	223	7	4.088	4.894	4.515	Exact time of conception known.
»	218	9	4.08	5.033	4.526	Parturition at full term.
»	152 _I	4	4.05	5.05	4.63	Exact time of conception known.
Diabetes, alloxan	207	8	4.523	5.60	5.082	Exact time of conception known.
Diabetes, pancreatectomy	209	8(9)	5.200	5.985	5.485	Parturition at full term
»	211	9	5.123	7.185	6.262	6.405±0.548 { Parturition at full term Exact time of conception known. Exact time of conception known.
»	195	6	5.73	6.84	6.45	
»	212	1		7.401	7.401	
Control	53	7(11)	3.45	4.20	3.84	4.661±0.553 (3.003—6.319)
»	168	9	3.715	4.876	4.379	
»	K	10	3.855	5.125	4.48	
»	78	8(10)	4.12	4.69	4.49	
»	125	4	4.15	5.32	4.79	
»	216	10	4.530	5.278	4.952	
»	128	9	4.665	5.655	5.149	
»	178	6	3.975	5.950	5.256	
Progesterone injection	144	6	5.244	6.385	5.845	3 days late.
Chorionic gonadotropin-injection	145	4	5.61	6.430	5.982	2 days late.

*) Only 5 infants of the whole litter (10) have been weighed.

cases. *Miller* describes in *Endocrinology*, April 1947, an investigation he carried out on the weight of the offspring of rats with diabetes produced by alloxan. He observed no giant growth in his experiments. My mother rats with diabetes due to alloxan also had no enlarged offspring. It is still too early to judge whether there is any difference, with respect to the size of the offspring, between alloxan diabetes and diabetes developing after pancreatectomy. In the case of diabetes following excision of the pancreas, also, only a few of the mother rats had offspring showing giant growth.

A number of hypotheses have been advanced to explain giant growth in some children of diabetic mothers. According to one view, the cause is hyperglycemia in the mother, possibly in conjunction with an increase in the supply of insulin to the fetus owing to hyperplasia of the islets of the fetus. The increase in weight has been compared with insulin fattening.

This attempt at an explanation cannot, in any case, be correct in general, since enlarged infants have been observed originating from mothers who have not had hyperglycemia. It is, moreover, not a question merely of increased obesity but of genuine giant growth. Of my 3 rats producing giant offspring 2 had high blood sugar values, but in one case (R 195) the values were only slightly raised, at intervals.

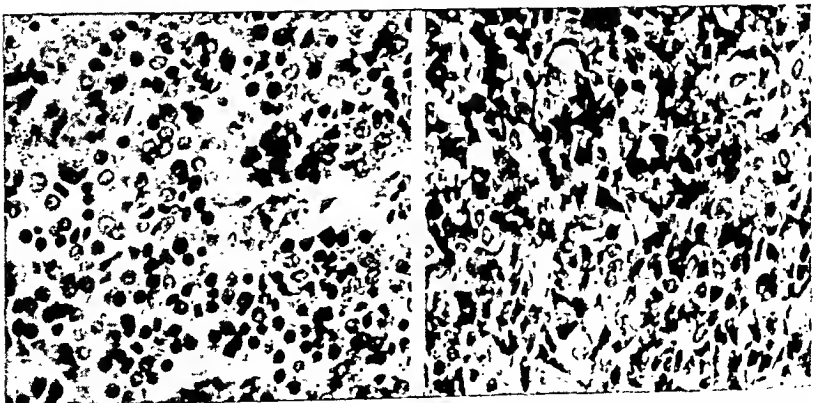
According to *Snyder* and *Hoopes*, the increased birth weight may be explained by the over-development of the fetuses as a result of prolonged pregnancy caused by an over-production of gonadotropin. In my experiments, the time of conception is known exactly in at least one of the cases of giant offspring (R 211), and in that case it was not a question of prolonged pregnancy. Nor is any particular prolongation of pregnancy likely to have occurred in the other two cases. For the sake of comparison, I have, in cooperation with *B. Engfeldt*, made a number of experiments with prolongation of the period of pregnancy in rats by means of injections of gonadotropin or progesterone towards the termination of pregnancy. Table 1 shows the weight of the offspring in two such cases with a prolongation of 2—3 days. The weights do not, by any means, reach those of the giant offspring. Nor do the experiences from pregnancy in diabetic women with large babies point to an essential prolongation of the period of pregnancy.

The giant growth has also been associated with the hypophysis. There are several reasons for assuming a connection between the diabetogenic effect of the hypophysis and its growth-promoting effect. The occurrence of diabetes in hypophyseal disturbances, above all in acromegaly, is well known. In animal experiments a diabetogenic effect by growth hormones has been obtained; the diabetogenic factor, however, does not appear, as was presumed at first, to be identical with the growth hormone, but is merely, in one way or another, coupled to the latter (*Young* and co-workers).

Table II.
Quantitative conditions in hypophysis of the rat infants.

	Infant no.	mm 3/g body weight	Volume of hypophysis				Average %		
			Ave- rage	Ant. lobe%	Mid. lobe%	Post. lobe%			
Control series	53,VII	0.0195	0.0248	74.8	12.7	12.5	75.6	12.2	12.2
	216,II	0.0275		78.8	11.2	10.0			
	216,IV	0.0216		75.5	11.7	12.8			
	216,V	0.0258		72.8	14.1	13.1			
	125,I	0.0254		73.5	11.8	14.7			
	78,III	0.0289		78.4	11.7	9.9			
Diabetes, giant growth	195,I	0.0231	0.0251	64.4	11.6	24.0	70.3	12.1	17.7
	195,V	0.0228		71.4	11.6	17.0			
	211,IV	0.0253		72.0	10.4	17.6			
	211,VI	0.0178		69.3	16.0	14.7			
	211,VIII	0.0339		72.0	11.4	16.6			
	212,I	0.0274		72.5	11.3	16.2			
Diabetes, normal size	207,I	0.0264	0.0298	78.4	9.5	12.1			
	209,IV	0.0332		68.3	15.7	16.0			
Progesterone injection	144,III	0.0181		71.3	10.8	17.9			
Chorionic gonadotropin- injection	145,IV	0.0206		73.9	11.7	14.4			

A qualitative and quantitative histologic examination of the hypophysis in the offspring in my material is now in progress, and I will here give some preliminary results. The volume has been determined planimetrically, as the hypophysis in a young rat is too small to be weighed exactly. The values are seen in table 2.



b

Fig. 3.

a

The anterior lobe of the hypophysis in young rats. a) control animal, b) animal from diabetes group, showing giant growth (R 211). Htx-Magdala red.

If the volume of the hypophysis is calculated per gram of body weight, no marked difference can be recognized between the control animals and the giant offspring. A certain increase in the volume of the hypophysis can be noted in a couple of normal-sized offspring of diabetic mothers. An interesting fact is that the volume of the hypophysis in those giant offspring produced through prolongation of the pregnancy by means of gonadotropin and progesterone shows fairly low values.

With regard to the proportion between the different lobes of the hypophysis, the giant offspring show a tendency to a decrease in the size of the anterior lobe, and an increase in the posterior lobe, whereas the midlobe remains constant.

So far, however, all these quantitative calculations are too few to justify definite conclusions.

The histologic picture shows significant differences between the offspring of the control animals and the giant offspring. The anterior lobe of the hypophysis in the control offspring appears more immature, with cells fairly poor in cytoplasm and with compact nuclei rich in chromatin. The hypophysis of the giant offspring shows greater vascularization and hyperemia. The cells are larger, and richer in cytoplasm, with distinct nucleoli. Fetal cell types are much less frequent than in the controls. A detailed study with special staining and a differential count has not yet been made, but judging by the preparations hitherto available, the cells appear to have reached a higher degree of maturity in the offspring with giant growth than in the controls, as regards the granulation also. Chromophilic cells occur in abundance and of these the basophilic cells are particularly predominant.¹⁾

The posterior and midlobes show changes in the cells similar in all essentials to those in the anterior lobe with a more differentiated cell picture.

Thus, definite changes in the cell pattern of the hypophysis were demonstrated in the giant offspring. There is the possibility of a modification in the proportions between the lobes but no changes in volume. To what extent these changes may be associated with the giant growth it is difficult to say, at least not until a further study has been made of the proportions between the cells, and until other endocrine organs have been examined.

¹⁾ In cooperation with *B. Engfeldt* I have, after the lecture, performed a histologic examination on the hypophysis of the large offspring produced by means of progesterone or chorionic gonadotropin injections in the mothers. The histologic picture of the hypophysis of the latter offspring showed a striking similarity to that seen in the giant offspring of diabetic rats, whereas the offspring of the rats injected with progesterone showed a more immature picture similar to that of normal young rats.

DISCUSSION

JØRGEN PEDERSEN: I should like to present some findings in studies in the blood sugar concentration in the first 24 hours of life in children of diabetic mothers. The material comprises 22 children of diabetics, 19 children of normal mothers. All the children were kept under identical and basal conditions throughout the first 24 hours, during which nothing was given them.

The curves for the average blood sugar values in the first 24 hours take fundamentally the same form in the children of normal mothers and diabetics. In the latter the blood sugar level was hardly 10 mg. % lower than in the children of normal mothers — in this series.

The blood sugar level in the children in the first 24 hours of life is directly proportional to the blood sugar values found in the mothers immediately before delivery.

ODDMUND KOLLER: I have been very pleased to hear the interesting paper given by Dr. Hultquist. During the last year and a half I have happened to occupy myself with the same problem and with similar methods — without knowing anything whatever about Dr. Hultquist's work. My experiments were carried out on white rats, and the pancreatectomy was performed at different junctures of the pregnancy. I have not made blood sugar determinations on my animals to the same extent as done by Dr. Hultquist. The diabetic state of the animals was watched by collection of the 24-hour urine, and the total sugar output was determined. None of my animals showed glycosuria except after pancreatectomy.

In the non-pregnant animals glycosuria appears about 7 days after the pancreatectomy, and then it keeps increasing gradually up to a level of 10—15 %, at which it then keeps for a varying length of time.

In the pregnant rats the postoperative glycosuria was less pronounced, 4—5 %. In contrast to Dr. Hultquist it has not been necessary for me to use insulin in order to make the rats go to the full term of pregnancy. None of my animals was treated with insulin. When the animals got over the postoperating trauma, they were thriving without insulin.

As to the 3 cases in which Dr. Hultquist thinks he has been able to demonstrate giant growth of the fetus, I have not been able to arrive at such a conclusion in my animals, in which the average weight of the newborn has been approximately 7 g. Besides, it is to be kept in mind that Dr. Hultquist has given insulin to his animals. The question then arises. What is due to the pancreatectomy, what to the insulin medication?

S. RANSTRÖM: A great deal of interest is attached to the observations reported by Dr. Hultquist about the appearance of the pituitary in the young ones born by rats in which the pancreas had been resected

previously. If the pictures presented of specimens of the pituitary from these young rats and from control animals really are representative, a differential count will hardly be required to convince the observer that here we are meeting with the real change in the pituitary. These experimental findings may be said to constitute the experimental analogy to previous observations on human material. For, fetuses of diabetic women show pituitary changes of fundamentally the same character as that described by Dr. Hultquist in his young rats, i. e. premature differentiation of the hypophyseal cells. In fetal erythroblastosis I have observed the same kind of changes in the pituitary. With reference to my paper, read yesterday, on the problem of erythroblastosis, I should like to ask whether any adrenal changes had been observed in these animals, and also whether there has been noticed any increase in the extramedullary hematopoiesis in these young rats.

G. HULTQUIST: Dr. Pedersen's studies on the behavior of the blood sugar of diabetic mothers are very valuable especially as, to my knowledge, they are the first systematic studies of this kind. In my experiments I have determined the blood sugar concentration on newborn rats and on their mothers during parturition and my findings here have been quite in keeping with those reported, as will be evident from Table 1.

Table 1.
Blood Sugar Concentrations in Percentage in Diabetic Female Rats and their Young.

	Rat number	Bloodsugar in % of			mother Average
		infants			
		Min.	Max.	Average	
Diabetes, pancreatectomy	198	0.031	0.048	0.041	0.072
»	209	0.076	0.118	0.090	0.129
»	218	0.086	0.152	0.120	0.132
»	223	0.095	0.144	0.128	0.162
»	214	0.099	0.122	0.109	0.173
»	211	0.116	0.171	0.148	0.280
»	152 II	0.330	0.347	0.338	0.349
» alloxan	207	0.290	0.372	0.331	0.402
Normal	78	0.075	0.108	0.085	0.095

From Table 1. it is evident that the blood sugar values in the newborn rats generally follow the maternal values, albeit in the somewhat lower level. In one case the maternal value was rather low, though not subnormal, and the values for the newborn were hypoglycemic (the normal value for this rat strain is about 0.080 %). A corresponding hypoglycemic reaction as observed in children of diabetic mothers could not be demonstrated in the rat experiments.

In connection with Dr. Koller's remark I should like to point out that we have to be very cautious in judging of diabetes in rats merely from the outcome of determination of the sugar in the urine. For there are several rat strains that have alimentary glycosuria which may give rise to mistaken conclusions if this fact is not kept in mind. Only blood sugar examinations can give quite reliable information.

Dr. Koller's and my own experiments appear to have been performed on different strains of rats. This is evident, among other things from the apparently higher birth weight in his material.

As to the significance of the insulin treatment to the appearance of giant growth in my experiments, I feel convinced that this has no particular influence on the results, partly for reasons already mentioned in my paper, partly because there was no demonstrable difference between cases with giant growth, in which insulin was given in small doses and cases with a large supply of insulin.

Examination of the adrenals and other endocrine organs has been commenced, but I am not able yet to give any account of the results obtained.

Comparative studies have been carried out also on the occurrence of erythropoiesis in the liver in the normal material and among the young of the diabetic rats. An abundant formation of red blood cells in the liver was found in both groups. Whether any distinct difference in this respect may be demonstrated I am not able to say, but so far no particular difference has been observed.

A CASE OF DIABETOGENOUS DWARFISM

By *Anders Bergstrand*.

There are a few cases described in the literature of so-called hypophyseal dwarfism in conjunction with diabetes mellitus (1, 2, 3, 4, 6, 7). Under the diagnosis of hypophyseal dwarfism appear to be included all cases of definitely subnormal body size without deformities of the skeleton, with normal proportions and well-developed intellect. In the clinical picture is also included a retarded or entirely lacking sexual development with poorly developed secondary sex characteristics. The cause of this disease is considered to be a hypofunction of the hypophysis cerebri as a result of trauma, congenital underdevelopment or tumours. The disease is thus closely related to Simond's disease and Fröhlich's syndrome.

In these diseases, however, the blood-sugar is as a rule low. Diseases which are due to a hyperfunction of the pituitary, e. g. acromegaly, are, on the contrary, often accompanied by diabetes mellitus. After Houssay's discovery of the diabetogenic hormone of the pituitary, many regard diabetes mellitus as a disturbance of the central regulatory mechanism hypothalamus-hypophysis, with a hypersecretion from the latter. It is thus difficult to explain the occurrence of diabetes mellitus in conjunction with a condition which depends on a hypofunction of the pituitary.

It has also been difficult to obtain morbid anatomical grounds for the assumption that a hypofunction of the pituitary is the basic cause of those cases of dwarfism which are called »hypophyseal«.

Engel (12) has recently, however, in a lecture to the Swedish Medical Association, demonstrated a number of cases of dwarfism with diabetes mellitus, one of which showed changes in the hypophysis cerebri. In general, these dwarfs react very badly to the administration of pituitary preparations containing growth hormones (Shelton) (16). Recently, therefore, the theory that a metabolic disease might be the primary cause of so-called hypophyseal dwarfism has been more prevalent. In those cases where diabetes mellitus has been present,

this would thus be considered as the primary disease which caused dwarfism in the patient.

I recently had the opportunity of making a post-mortem examination of such a case, in which the clinical history and post-mortem findings could possibly illuminate these problems.

The case in question was a young woman, who died at the age of 27. Her development had been normal up to the age of three when, following mild poliomyelitis, it was found that she suffered from diabetes mellitus. She was treated at home by dieting and small doses of insulin. She had occasional mild attacks of precomatose type and was admitted to hospital in this condition in April 1939.

She was then 18 years old, 125 cm. in height, and weighed 28 kg. Her body was well-proportioned. The epiphyses, for example in the tibia, showed insignificant ossification. She had not yet menstruated. The blood-sugar was 400 mg. %. Legal's test was positive and there was 1.3 % sugar in the urine. B. M. R.: —8. No other endocrine disturbances. She had gone through the primary school and was considered talented. Her I. Q. was 105.

Great difficulty was experienced in regulating her diabetes on account of her very low carbohydrate tolerance and sudden changes in the percentage of blood-sugar. The possibility of attempting treatment with pituitary extract was considered, but abandoned in view of the age of the patient and the great difficulty of obtaining such extract.

The patient was re-admitted, once more in precoma, in the autumn of 1943. Her height was then 129 cm. She had menstruated, but menstruation was irregular. Information regarding secondary sex characteristics is lacking. She was then treated in the Out-Patients' Department. In April 1944, she complained of vertigo and headache. Her blood-pressure was found to be 165/115. Heller's test was positive, and there were abundant white corpuscles in the urine. Water tests showed poor excretion and concentrating capacity. At the same time, a macrocytic anaemia was found. Later in the same year the blood-pressure was 175/125.

Since 1943 she had dark, progressive deterioration of sight, and in January 1946, she was nearly blind, owing to bilateral cataract. The blood-pressure was then 175/110. She came to the hospital on account of amenorrhoea for two months. The uterus was somewhat enlarged, and in March of the same year an abortion was induced in the fifth month. Her general condition was thereafter good, but her blood-pressure rose slowly. In April 1946, it was thus 230/150.

On April 9th, 1947, she visited the hospital in order to obtain a certificate for admission to a school for the blind. When the doctor in the Out-Patients' Department did not consider that he could write this in the way she wished, she had a violent hysterical attack, and went away. About two hours later she was brought back to the hospital in very poor condition, and died with a clinical picture of pulmonary oedema before any therapeutic measures could be taken.

Post-mortem examination revealed a young woman, of proportionate body size, 132 cm. in height. The neck was strikingly short and the head appeared somewhat large in proportion to the rest of the body. The facial features gave the impression of considerably greater age than was the case (See Fig.). The secondary sex characteristics were well developed and her bodily configuration was typically feminine without virile traits.



The internal organs showed general, very severe arteriosclerosis. In addition, the kidneys were very shrunken (total weight 135 g.). On microscopical examination this proved to be due to arteriolosclerosis. The heart showed considerable hypertrophy of the left ventricle.

The hypophysis cerebri weighed 0.56 g. which, considering the body size and weight of the patient, appears normal. Microscopical examination showed no pathological cell forms. The number of chromophil cells appeared large in relation to the number of chromophobe cells, and the eosinophil cells in particular were numerous, in opposition to statements in reports of cases published earlier, in which an increase of the number of basophil cells was found. (Engel: investigation by Mellgren).

There was considerable fibrosis and arteriosclerosis in the pancreas. It was remarkable that the normal islets were entirely lacking. Here and there, however, it was possible to find remains of islets,

partly in the form of single cells and partly in the form of large groups of insular cells.

The uterus was normal in size, with the mucosa in the transition between proliferation and secretion phases. The ovaries were also of normal size and contained numerous ripe follicles, as well as a fresh corpus luteum on the right side. The other endocrine organs showed nothing remarkable. The lenses of both eyes were totally clouded and the retina atrophic. Examination of the brain, the basal ganglia, the brain stem and the hypothalamus region [nuclei supra-optici et paraventriculares] showed no lesions except a few microscopical areas of softening probably caused by the hypertension. The primary cause of death appeared to be acute cardiac insufficiency and pulmonary oedema.

The clinical picture of the patient, with its many symptoms, can be discussed from many standpoints. In this paper, however, only the question of the importance of diabetes will be discussed. In the earlier stage of the disease, the picture conforms satisfactorily with a so-called hypophyseal dwarfism. The latter phase, on the contrary, differs considerably from the usual course. With the exception of delayed puberty and irregular menstruation, no defects of the functions of the sex organs were observed. The patient had thus reached full sexual maturity and was even capable of conception. The autopsy revealed a hypophysis cerebri without definite pathological changes, in addition to well-developed sex organs and functioning ovaries.

In my opinion, there is thus nothing in this case which indicates that the primary cause of the patient's dwarfism was a hypofunction of the hypophysis cerebri. I thus interpret the disease as a juvenile diabetes, very difficult to treat, with low carbohydrate tolerance, and in consequence subnormal bodily growth and retarded development.

Summary.

The author reports a case of dwarfism in a young woman, suffering from a diabetes mellitus since the age of three. She developed severe hypertension and arteriosclerosis and died at the age of 27 from heart failure.

The autopsy revealed no signs of a hypofunction of the pituitary. The young woman reached full, though somewhat delayed, sexual maturity and was even capable of conception.

The author regards the diabetes mellitus as the primary cause of the dwarfism.

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DISCUSSION

ASK-UPMARK: Pathologic-anatomically there is nothing to be added to the account of this interesting case. From a clinical point of view, however, I wish to call attention to three points to be added to Dr. Bergstrand's paper:

For one thing, the development of diabetes in connection with poliomyelitis gives rise to many considerations.

In the next place the unfortunate lack of clinical data in the case record concerning such an important matter as the secondary sex characters.

In the third place the death of the patient from pulmonary edema, which undoubtedly may be ascribed to her troubles with the Out-patient Clinic — that this poor woman was denied the desired and well-motivated certificate is not only outrageous, I think, but also contributory to her death.

PROBLEMS CONCERNING THE ESTIMATION OF THE CHEMOSENSITIVITY OF MICROBES AND MEASURING OF PENICILLIN AND STREPTOMYCIN CONCENTRA- TIONS IN THE BLOOD AND SPINAL FLUID

By K. A. Jensen and Inger Kiær.

I should like briefly to outline a rapid method for determination of the resistance of bacteria present in specimens of clinical material — as, for instance, in cases of infection of the urinary tract.

The urine is centrifuged, and a smear is stained after Gram.

If this smear shows at least a few bacteria in each field, a direct determination of their resistance may be made as follows.

On a blood agar plate (ab. 14 c. c. in diameter) six sterile pieces of filter paper, 2 c. c. in diameter, are placed at a suitable distance from each other. The first piece of filter paper is moistened with 1/20 c. c. of a streptomycin solution containing 2800 units per c. c., the second with 1/20 c. c. of sulfathiazole solution of 6,6 mg per c. c., the third with 1/20 c. c. of penicillin solution containing 175 units per c. c., the fourth with streptomycin + sulfathiazole, the fifth with streptomycin + penicillin, and the sixth with sulfathiazole + penicillin.

After standing for ten minutes the filter paper is removed, and the plate is allowed to dry for a short time.

Then one loopful of the urinary sediment is spread on each of the areas (see fig. 1), and the plate is incubated at 37° till the next day, when it shows directly whether the bacteria present in the urine are inhibited in their growth by streptomycin, penicillin, sulfathiazole or any of the three combinations.

In this way it is possible within 24 hours to tell the clinician which remedy is likely to be the most suitable in the given case.

In our hands this method has given excellent results — not only when applied to specimens of urine, but also to other materials. If the bacterial content of a given specimen is very slight, it may be advisable to make a preliminary culture in a fluid medium. In addition it is possible to make a rough classification and pure cultures by mi-



that we are able to measure even less than 3 units per c. c. by the agar-cup method, while the other strain is so sensitive to penicillin that we are able to measure about 1/15 Oxford unit per c. c. in this way.

For measuring of the streptomycin contents of serum we fill four cups respectively with 25, 12,5, 6,25 and 3,12 units of streptomycin

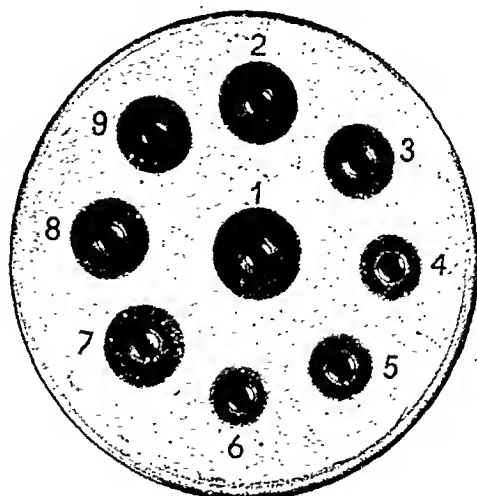


Fig. 2.

1 =	Standard Streptomycin	25	Units
2 =	»	12,5	»
3 =	»	6,25	»
4 =	»	3,12	»
5 — 6 — 7 — 8 — 9 =	Serum		

per c. c. In the remaining cups we place undiluted patient serum or spinal fluid. On the following day the diameters of the inhibition-zones are measured (see fig. 2), the curve for standard streptomycin is drawn, and thus it is possible to calculate the potency of the various specimens in question (see fig. 3).

The measuring of the penicillin blood level is carried out in the same manner. (See fig. 4). In this way we have measured 6—8 blood samples on the same agar plate.

The results thus obtained agree very well with the dilution method. See table 1 and 2.

Inactivated serum and spinal fluid has been used for the dilution method.

Using the agar-cup method the result has shown to be the same with inactivated and active serum and spinal fluid.

Using our strain in the agar-cup method we have never seen any inhibition-zone with normal serum and spinal fluid.

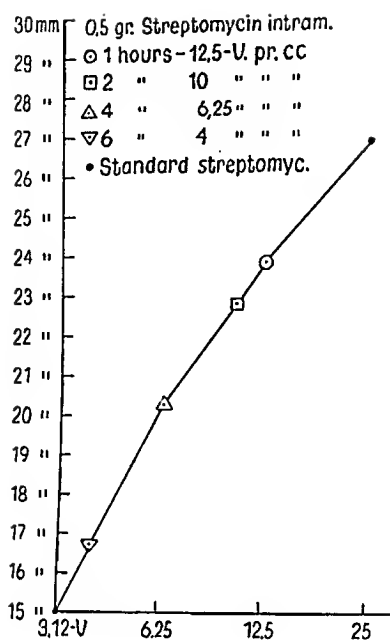


Fig. 3.

Table 1.

The concentration of streptomycin in blood and spinal fluid determined by the agar-cup method, and the serial dilution method.

		Agar-Cup Method	Dilution Method
Serum	1	22.5 U. per. c.c.	20 U. per c.c.
"	2	20.9 " " "	16 " " "
"	3	15.5 " " "	16 " " "
"	4	29.4 " " "	32 " " "
Spinal fluid	1	20 " " "	20 " " "
Serum	5	19.6 " " "	18 " " "
"	6	12.5 " " "	12 " " "
"	7	7.8 " " "	8 " " "
"	8	11.0 " " "	14 " " "
"	9	6.9 " " "	8 " " "
"	10	8.7 " " "	10 " " "
Spinal fluid	2	7.3 " " "	8 " " "
Serum	11	17 " " "	15 " " "
"	12	14.9 " " "	14 " " "
"	13	8.4 " " "	9 " " "
Spinal fluid	3	17 " " "	16 " " "
Serum	14	12.5 " " "	12 " " "
"	15	11.6 " " "	12 " " "
"	16	10.9 " " "	12 " " "

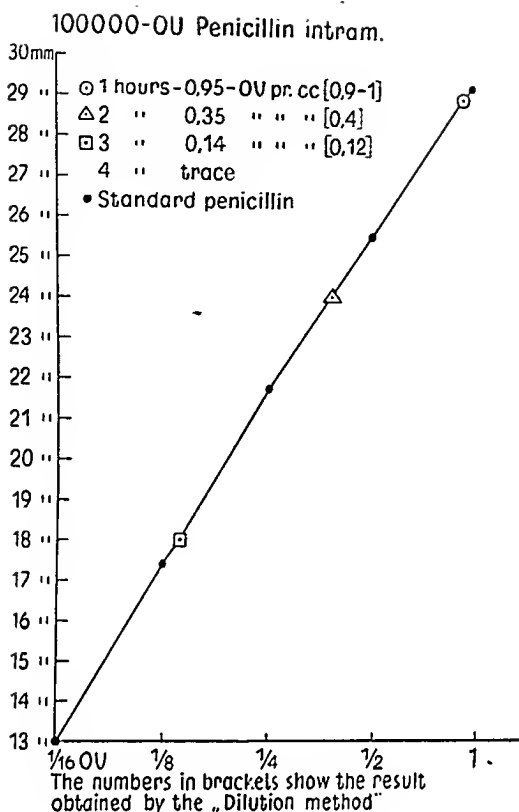


Fig. 4.

Table 2.

The concentration of penicillin in blood determined by the agar-cup method and the serial dilution method.

	Agar-Cup Method	Dilution Method
Serum 1	2.5 O. U.	2.54 O. U.
" 2	0.83 "	0.56 "
" 3	0.65 "	0.48 "
" 4	0.63 "	0.48 "
" 5	0.63 "	0.48 "
" 6	0.22 "	0.22 "
" 7	0.19 "	0.16 "
" 8	0.07 "	ab. 0.05 "
" 9	trace	" 0.04 "
" 10	"	" 0.03 "
" 11	"	" 0.02 "
" 12	0.95 O. U.	0.9-1.0 "
" 13	0.35 "	0.40 "
" 14	0.14 "	0.12 "
" 15	trace	ab. 0.05 "

In undiluted serum and other body fluids in this way it is possible to measure from 1 to 50 units of streptomycin, and the streptomycin blood level is usually found to lie within this interval; as to penicillin we are able in this way to measure from $1/15$ to 1 Oxford unit. With higher penicillin blood levels the serum has to be diluted once or more times.

TOXIN-FORMING STAPHYLOCOCCI, AS CAUSE OF DEATHS ON THE INJECTION OF INFECTED BACTERIOLOGICAL PREPARATIONS

By G. Olin and A. Lithander.

At Bundaberg in Australia, in 1928, several deaths occurred after the injection of a diphtheria vaccine which had been contaminated with staphylococci. The vaccine, which consisted of a mixture of diphtheria toxin and antitoxin without the addition of an antiseptic, had been kept in the original packing, provided with a piercable rubber cap. The bottle was first used on three different occasions in the course of five days without giving rise to any abnormal reactions to the inoculation. When the same bottle, which had been kept at room temperature, was used again three days later for the inoculation of 21 children, 16 of them, 5—7 hours after the injection, fell ill with vomiting, diarrhea, high fever and in most of the cases unconsciousness, cyanosis and convulsions. Twelve of the children died 15—34 hours after the injection. In all the children that survived, an abscess appeared at the place of the injection. From these abscesses as well as from the vaccine, staphylococcus aureus with the same characters was cultivated.

This calamity at the time caused a great sensation and occasioned a thorough investigation by a commission, whose members were Kellaway, Mac Callum and Tebbutt.

This inquiry issued in the conclusion that the cause of the cases of death and disease must be attributed to the injection of the diphtheria vaccine. The presence of free diphtheria toxin could be ruled out. In the vaccine, however, staphylococcus aureus could be found in pure culture. This staphylococcus proved to be identical with staphylococci cultivated from abscesses at the place of injection in 5 examined surviving children. Its pathogenicity was small for guinea-pigs, mice and monkeys. Intravenous injection in rabbits, however, led to death within 12—24 hours, with convulsions, but without macroscopic postmortem changes. The staphylococcus was weakly hemolytic and fermented mannitol. When grown in broth, it produced within 7—8 hours a toxin which was rather labile, but which in man could induce cutaneous reactions even when diluted down to 1/2,000.

According to the official report, all the facts indicated that the Bundaberg calamity had been caused by an injection of a diphtheria vaccine containing live staphylococci. As regards the pathogenesis, it was supposed that the violent symptoms and the deaths were mainly attributable to a toxemia induced by a copious formation of toxins resulting from the growth of the staphylococci at the place of injection.

In Sweden during the course of the last few years, in close connection with injections of bacteriological preparations, three deaths, in all, have occurred among children. This happened on two different occasions.

Two of these fatalities occurred in 1945 after injection of measles convalescent serum prepared at a hospital laboratory. Three children then simultaneously received intramuscular injections of this serum from the same bottle, and fell ill, with toxic symptoms, shortly after the injection. Two of them, aged 11 and 8 years, respectively, died 13¾ and 17½ hours afterwards. The clinical picture as well as the postmortem findings pointed to acute toxic damage. Within 6—8 hours after the injection all three children were affected with vomiting, diarrhea and high fever. Cerebral symptoms in the form of somnolence and agitation were manifested. Cyanosis soon supervened, and two of the children died a few hours later. The third child presented the same disease picture, but nevertheless survived.

The postmortem examination of the two children showed very large and extensive cadaveric stains. In the liver, degeneration with small fat droplets was observed, and in the kidneys a cloudy swelling of the convoluted tubules, i. e. parenchymatous degeneration indicative of toxic action (Sjövall).

After the treatment of these three children, the contents of the serum bottle were practically exhausted. The same bottle had been used 8 months before, without any undesirable effects, for injections given to several patients. The bottle had afterwards been kept in a refrigerator. The serum contained quinosol, and formalin as preservatives. From this serum bottle, a strain of staphylococci, in the sequel termed »Lethal«, was cultivated. From another bottle, from which serum had been used for the prophylactic treatment of eleven other children, who had not shown any toxic symptoms, a staphylococcus, in the sequel termed »Non-lethal«, was isolated.*) Both serum bottles, before the bacteriological examination, had stood uncorked in room temperature over night.

The third fatality occurred at a hospital for children in 1946. It was the case of a one-year-old child, who had been subjected to a Mantoux test of 1 mg tuberculin intracutaneously. Four hours later the child suddenly fell ill, with vomiting, diarrhea and high fever.

*) These strains were kindly supplied by Professor A. Lindau.

After a progressive change for the worse, with spells of unconsciousness and circulation failure, the child died 17 hours after the injection, although penicillin treatment was introduced about 1½ hours before the death.

At the postmortem staphylococcus aureus was isolated from the local lesion, which had an extent of 20×20 mm with a blue-discoloured vesicle in the centre (strain »Local«), as well as from a regional lymphadenitis in the axilla (strain »Axilla«). Ten other children were simultaneously subjected to Mantoux tests with the same tuberculin solution, and in most of them similar toxic symptoms resulted, in some cases severe, but without lethal issue. From the local lesions in two of these ten children, staphylococci were likewise isolated (»Pat. H.« and »Pat. F.«). From the tuberculin solution used, strains »Tub. hem.« and »Tub. anhem.«*) were isolated.

The tuberculin solution, which had been prepared at the hospital, did not contain any preservative and had been kept in a refrigerator for about a week, but, in connection with the consulting-hours at the out-patient department, had stood at room temperature for several hours daily. After the immediately preceding use of this solution no abnormal reactions had been observed. All the staphylococci strains, except »Tub. hem.«, coagulated plasma and fermented mannitol, characters indicative of pathogenicity.

From all the above-mentioned strains toxin was prepared, in accordance with the technique described by Lithander (1944) with semi-solid media in an atmosphere composed of 25 % CO_2 and 75 % air.

The formation of toxins was examined firstly by inoculation direct from the original cultures, and secondly after passage through rabbits for the purpose of retaining the virulence. The culture was incubated about 40 hours. After control of the purity of the culture, the entire medium was centrifuged in order to separate the agar. It was then filtered through paper and a Seitz filter. The filtrate was mixed with merthiolate to a concentration of 1/10.000. After sterility control, the strength of the toxin was tested firstly in vitro, and secondly in rabbits. With each strain, several toxin preparations were made. Each time the »Lethal« and »Non-lethal« strains were used, both strains were inoculated on the same batch of medium. The two toxins were thus prepared under identical conditions. Also in regard to the strains isolated in connection with the third fatality, the formation of toxins was tested simultaneously for several strains on the same medium.

Titration of the toxins in vitro.

The direct toxic value (MHD = minimum hemolyzing dose) was determined for all the toxins against rabbit and sheep cells as well as for some of the toxins against human red cells. With rabbit cells, moreover, the indirect toxic value (L_h) was determined against standard serum from the State Serum Institute in Copenhagen.

*) Facilities for the examination of these two strains were kindly provided by Professor H. Davide, who will report his examinations elsewhere in connection with a full description of the clinical picture of the cases.

In all the tests, 2 % suspensions of washed red cells were used. In determining the MHD, readings were taken after 1 hour at 37°, for sheep cells also after 20 hours at 4°. The titre was read for traces of hemolysis (10 %), as regards rabbit cells for complete hemolysis and, in certain cases, also for 50 % hemolysis. The results are shown in Table 1. In determining the L_h dose, which was done with rabbit cells, readings were taken after 1 hour at 37° and 1 hour at room temperature.

Titration of the toxins in vivo.

Rabbits weighing about 2.5 kg were used. The lethality of the toxins after intravenous injection was examined, as also their dermonecrotizing effect after intracutaneous injection (MND). The intravenous injections were dosed according to the body-weight of the rabbits; in the intracutaneous injections 0.1 ml of the toxin or its dilutions was injected. The results are shown in the following Table 2.

Table 1.

Strain	M H D Rabbit cells hemolysis			M H D Sheep cells hemolysis 10 ⁶ 10 ⁶ 1 h. 37° 20 h. 4°		M H D Human cells hemo- lysis 10 ¹⁰		Indirect toxic action L_h dose
	100 %	50 %	10 %					
Non-lethal			0.1—1.5	0.1—1.0	0.1—1.0	1.0		2
Lethal	0.2—0.007	—	0.005— 0.0007	0.05— 0.03	0.03— 0.005	0.05		0.50—2
Local and	0.007—	0.001—	0.0005—	0.007—	0.003—	0.7	0.25	0.10
Axilla	0.001	0.0002	0.0001	0.001	0.0001			0.22
Tub. anhem.	0.007—	0.002—	0.001—	0.005—	0.007			0.10
and hem.	0.0002	0.0003	0.0001	0.001	0.0005	0.7		0.33
Pat. II. and	0.007—	0.002—	0.0005—	0.007—	0.003—	0.7	0.25	0.10
F.	0.0007	0.0002	0.00005	0.001	0.0007			0.33
Wood and	0.002—		0.0001—					0.05
Walker	0.0005		0.00001					

From Table 1 it will be seen that the strain »Non-lethal« has a low direct toxic value, judging by its hemolytic action on rabbit, sheep and human red cells (MHD), and also a relatively low indirect toxic value (L_h dose). The strain »Lethal«, on the other hand, shows a relatively high direct and indirect value, differing in this respect from other strains of staphylococci examined at the laboratory, having been isolated from cases of clinically severe skin infections. Still more marked is the formation of toxins isolated from, or in connection with, the third fatality (»Local«, »Axilla«, »Tub. anhem.«, »Tub. hem.«, »Pat. II.« and »Pat. F.«). Particularly strong is the hemolytic effect of the toxin on rabbit and sheep cells, whereas it is relatively moderate against human red cells. No difference between these 8 strains can be found with respect to their toxicity. These strains have practically the same extremely potent toxin-forming capacity as the two international strains of staphylococci, »Wood 46« and »Walker«, which are in general use for the preparation of toxoids.

Table 2.

Strain	MND	Survival time in hours after intravenous inj. in rabbits (ml/kg)						
		3	3	1	0.5	0.3	0.2	0.1
Non-lethal	0.1	$\frac{4}{1}$	$\frac{5-22}{2}$ $\frac{s}{2}$	$\frac{0.25}{1}$ $\frac{17}{1}$	$\frac{s.}{1}$			
Lethal	0.003— 0.001			$\frac{0.5-2.75}{3}$ $\frac{4-16}{1}$	$\frac{4-20}{3}$ $\frac{s.}{1}$			
Local and Axilla	0.0001— 0.00001					$\frac{1}{1}$	$\frac{1}{2}$	$\frac{1.3}{2}$ $\frac{1.5-14}{3}$ $\frac{s.}{1}$
Tub. anhem. and hem.	0.003— 0.00001						$\frac{1}{1}$	$\frac{2.5-4.5}{3}$ $\frac{6-20}{1}$ $\frac{27}{2}$
Pat. H. and F.	0.0003— 0.00001							$\frac{1.5-5}{2}$ $\frac{1.5-20}{2}$
Wood and Walker	0.0002— 0.00003							$\frac{0.25}{10}$

The numerator indicates the number of hours, the denominator the number of animals. (s. = the animal survived).

The results of the in vivo tests are seen in Table 2. The strain »Non-lethal« showed a very slight dermonecrotic effect; merely with one of the toxins could traces of necrosis be elicited. In large doses, however, these toxins in certain cases had a lethal effect after intravenous injection in rabbits. Toxin from »Lethal« has a much more powerful action both in regard to dermonecrosis and the lethal effect on rabbits. Still more powerful are the toxins prepared from the strains isolated in connection with the Mantoux-test fatality. In regard to dermonecrotic capacity, they are quite comparable with toxins from the strains »Wood 46« and »Walker«, whereas larger doses were required to produce lethal effects by intravenous injections in rabbits than was the case with toxins from the two last-mentioned strains.

At the necropsy of rabbits whose survival time had been long enough to permit macroscopic changes to appear, it was found that these changes were the same for all the tested staphylococci. The changes were most marked in the kidneys, where they consisted in cloudy swelling, hyperemia and minor hemorrhages. In the intestinal walls and liver, hyperemia was often observed. The adrenals rather often showed slight changes in the form of hyperemia.

Tests against α - and β -antitoxins, both in vivo and in vitro, showed that the toxins prepared from all strains consisted mainly in α -toxin.

When live staphylococci had been passed through rabbits, which was done in immediate connection with the preparation of the toxins, the rabbits, almost without exception, died within twenty-four hours. It is noteworthy, however, that strain »Tub. hem.« on two occasions did not kill the rabbits, despite the injection of large numbers of bacteria. It may be recalled that this strain, unlike the others, did not coagulate plasma nor ferment mannitol. A feature common to all the strains is that their toxic effect is not modified by passing through animals.

As regards the Bundaberg fatality, the conclusion of the Commission was that the deaths had been caused by the injection of live staphylococci present in the diphtheria vaccine. The toxin-forming capacity of the Bundaberg strains, unfortunately, cannot be directly compared with that of the strains described here, as the present technique for the production of toxins is superior to that adopted for the Bundaberg strains. It should moreover be noted that other methods for determining the strength of the toxins had been applied in testing the last-mentioned strains.

The three above-mentioned fatalities in this country may be said to be very similar to the Bundaberg cases. Strain »Lethal« and the strains isolated from the tuberculin-tested cases as well as from the tuberculin dilution show a very marked toxigenic capacity. In view of this capacity, it may be considered that the staphylococci found are a sufficient explanation of the deaths. The fatal issue should be attributed mainly to the production of toxins in vivo during the growth of the bacteria but also to the introduction of toxins preformed in the injected fluid. The latter seems to be the case especially as regards the deaths after the injection of convalescent serum, when a large amount had been injected.

At a very early stage, symptoms from the alimentary canal, vomiting and diarrhea, were observed in the patients. It might seem plausible to assume that these symptoms, which appear to resemble those in food poisoning or acute gastroenteritis, may have been due to an enterotoxin effect. This, however, does not seem to be the case: these effects should be regarded as subsidiary symptoms in the general picture of toxic disease. In fact, vomiting and diarrhea occurred to a considerable extent also in the Bundaberg cases. The commission of inquiry pointed out the above-mentioned resemblances to the symptoms in food poisoning or acute gastroenteritis, but did not attach special importance to these symptoms, as effects of this nature often occur after the injection of any toxin whatever. Similar symptoms manifested themselves also in the rabbits tested, as above described, in regard to the in vivo effect of the toxins. That symptoms from the alimentary

canal, particularly diarrhea, occur as subsidiary effects of intoxications — induced by intravenous injections — with staphylococcal, streptococcal, meningococcal and diphtheria toxins, has been shown also by Lithander (1945).

As above mentioned, the infected tuberculin dilution that gave rise to toxic staphylococcal infections had not been admixed with any preservative. It accordingly seemed to be of interest to ascertain whether any of the most commonly used preservatives — quinosol, carbolic acid and merthiolate —, could kill the staphylococci in question, in a tuberculin dilution. The tuberculin was diluted to 1/100, firstly with physiological saline, secondly with a phosphate buffer solution, and the above-mentioned preservatives were added. After inoculation of 11.000—40.000 staphylococci, the tubes as well as controls were kept at 37°, 22° and 4°, respectively. The results are shown in Table 3.

Table 3.
Inoculation of staphylococcal strain »Local«.

Tuberculin dilution	40.000 cocci			11.000 cocci			20.000 cocci		
	37°	22°	4°	37°	22°	4°	37°	22°	4°
with phys. NaCl 1/1000	+ ¹	+ ²	+	+ ¹	+ ²	+	+ ¹	+ ²	+
without preservative	—	—	—	—	—	—	—	—	—
with 0.1 ‰ quinosol	—	—	—	—	—	—	—	—	—
with 0.5 ‰ »	—	—	—	—	—	—	—	—	—
with 0.5 ‰ carbolic acid	+ ¹	+ ⁸	+	—	—	—	—	—	—
with 0.1 ‰ merthiolate	—	—	—	—	—	—	—	—	—
with phosphate buffer	25.000 cocci			11.000 cocci			20.000 cocci		
without preservative	+ ¹	+ ²	+	+ ²	+ ³	+	+ ²	+ ³	+
with 0.1 ‰ quinosol	—	—	—	—	—	—	—	—	—
with 0.5 ‰ »	—	—	—	—	—	—	—	—	—
with 0.5 ‰ carbolic acid	—	—	—	—	—	—	—	—	—
with 0.1 ‰ merthiolate	—	—	—	—	—	—	—	—	—

The index figures after the plus sign indicate the number of days when macroscopically visible cloudiness had been observed.

In one of the tests it was found that the relatively large inoculation of 40.000 staphylococci was not completely killed by 0.5 % carbolic acid. Smaller amounts, however, were eliminated by this percentage. Quinosol 0.1 ‰ and 0.5 ‰ as well as merthiolate 0.1 ‰ had a complete sterilizing effect in all the tests, irrespective of the temperature.

Corresponding tests were made also with horse and human serum; the effect of formalin was then likewise investigated. The results are shown in Table 4.

Table 4.
Inoculation of staphylococcal strain »Local«.

	16.000 cocci					25.000 cocci					18.000 cocci					25.000 cocci				
	Human serum					Horse serum					Horse serum					Horse serum				
	37°	22°	4°	37°	22°	37°	22°	4°	37°	22°	37°	22°	4°	37°	22°	37°	22°	4°	37°	22°
without preservative	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
with 0.1 ‰ quinosol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
with 0.5 ‰ »	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
with 0.5 ‰ carbolic	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+
with 0.1 ‰ merthiolate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
with 0.2 ‰ formalin	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
with 0.4 ‰ »	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

In this case too it was found that one of the tests with carbolic acid showed defective capacity for sterilization, namely in regard to horse serum. The other preservatives, on the other hand, in all cases completely killed the staphylococci. In order to ascertain whether the effect of these agents weakened with time, the serum tubes were re-infected one week later in a test with 44.000 staphylococci. Forty-eight hours afterwards, however, these cocci too had been killed.

Summary.

Three cases of lethal staphylococcal infections are reported, two of which had resulted from intramuscular injection of measles convalescent serum, one from an intracutaneous injection of a tuberculin dilution. In both instances the injected material was infected with staphylococcus aureus, with a very high toxigenic capacity, which was studied in detail. It was shown that most of the preservatives commonly used in bacteriologic products could kill the staphylococci in question.

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DISCUSSION

O. RENKONEN: In Finland we have observed a similar case, in which convalescent plasma after measles was employed by a general practitioner without any injurious result whatever. 3 weeks later the same plasma bottle was used again — this time for the treatment of 3 children, 2 of whom died while the third was saved by intensive penicillin therapy. On analysis the plasma was found to be infected with

yellow, mannite-positive, coagulase-positive, hemolytic staphylococci. Unfortunately the plasma had not been disinfected with the mercurial preparations usually employed (merthiolate).

T. VOGELSANG: I have noticed that the children have had diarrhea. Indeed, *Staphylococcus aureus* is able to produce a number of different toxins. So, I think, it seems not unlikely that besides the lethal and hemolytic toxin, also the enterotoxic-toxin may have played a rôle. In this connection, mention could probably be made of a child suckling its mother who had a staphylococcus mastitis. The child died of enteritis, and the strain of staphylococcus aureus was isolated from the intestinal canal. The toxin produced by this strain caused vomiting and diarrhea in kittens.

My assistant Dr. Arlberg, has isolated 9 additional strains from cases of staphylococcus mastitis and 6 of these strains likewise produced enterotoxin that was demonstrated by experiments on kittens.

INFECTION WITH *ACTINOMYCES MURIS RATTI* AFTER A RAT BITE

By L. O. Borgen, M.D.

Infections after rat bites are not rare. When a patient is stricken after having been bitten by a rat, the examination is usually conducted with two diseases in mind, namely Sodoku or rat bite fever caused by *Spirillum minus* as described by Futaki and co-workers in 1916, and Weil's disease, caused by *Spirochaeta ictero-hæmorrhagiae*, described in 1916 by Inada, Ido, Hoki, Kaneko and Ito. The disease described in the present paper resulting from the bite of a laboratory rat is much more rare.

Case history.

A 25 year old woman, laboratory assistant, daughter of a physician, was bitten by a laboratory rat on the third finger of the left hand on Dec. 5, 1946. She had previously been bitten several times without becoming ill. During the two following days there were signs of local infection as the back of the left hand was red and swollen, accompanied by tenderness in the lower arm and axilla. Her father, who was a physician, treated her with sulfathiazole and on the 10th of December she felt so well that she resumed her work. However later in the day she felt ill and went home. That night she became gravely ill, had persistent chills, nausea, headache and slight pains in the back.

On Dec. 11, 1946, she was admitted to the Medical Dept. (VIII) of Ullevål Hospital, chief Carl Müller, M.D.

Status præsens Dec. 11, 1946.

The patient has flushed cheeks, complains of headache. Tp. 40.2° C. Third finger of left hand, a small, healed wound. Very slight reaction around wound. No local gland swelling. No exanthema. Examination of the internal organs revealed nothing.

The diagnostic considerations on admission were rat bite fever, leptospirosis or eventually a pyogenic infection.

The assistant physician of the Dept., Dr. Victor Gaustad, conferred with me about the case by telephone, and blood samples were taken for examination before treatment with sulfathiazole and pencil-

lin was begun. It should be stated that the patient recovered rapidly under this treatment. In this connection I would like to mention that this case, both clinically and bacteriologically, will be published in English translation in *Acta Medica Scand.* by the author and Dr. Gaustad. The present paper does not allow time to describe the case in detail so anyone who is interested will find all the details later in the above-mentioned paper.

Before I discuss the bacteriological investigation it may be mentioned that it revealed an affection not previously diagnosed in Norway (Scandinavia?), namely, rat bite fever caused by *Actinomyces muris ratti* sive *Streptobacillus moniliformis*. A brief historical discussion of this microbe will be presented in conclusion to the bacteriological examination. Rat bite fever caused by *Act. muris ratti* and rat bite fever caused by *Spiril. minus* are clinically very similar. Osler's textbook of 1944 states: »the disease presents the unusual feature of having identical animal reservoirs, identical epidemiological spread and identical symptoms, but different etiologies«.

Albritten, Sheely and Jeffers, 1940, present the *differential diagnostic features* of the two diseases. They point out the following: In the *Act. muris ratti* infection one finds *arthritis* and a *rash* with *small*, individual efflorescences, — in the *Spirillum minus* infection there is seldom arthritis and the rash exhibits *large* individual efflorescences. The *incubation period* for the *Act. muris ratti* infection is very short — for the *Spirillum minus* infection it is from 7 to 21 days or longer. The fever is more irregular in the *Act. muris* infection, while in the *Spir. minus* infection the fever is more regular and recurrent.

But on the whole the differential diagnosis must always be confirmed by the *bacteriological* examination. This is very important for the treatment also, as penicillin is very effective against the *Act. muris* infection, but not against the *Spirillum minus* infection. The reverse is true of the arsenic preparations as these are excellent in the latter disease, but ineffective in the former.

It is of interest that *Act. muris ratti* infections may occur through food. Infection through milk has occurred, as *Parker and Hudson* in 1926 found *Act. muris ratti* s. *Streptobacillus moniliformis* as the cause of an epidemic of 86 cases in Haverhill, Mass. This disease is called Haverhill fever or erythema arthriticum epidemicum and the authors called the *Act. muris ratti* microbe *Haverhillia multiformis*.

On Dec. 11, 1946, the Bacteriological Laboratory of Ullevål Hospital received a citrate blood sample from the above described patient with a request to have it examined for *Mb. morsus muris* and *Mb. Weil*. The ordinary bacteriological examinations for these diseases gave no results.

However a number of cultures had been made with the possibility of other infections in mind, and a blood broth culture gave growth

after 3 days. There was growth of typical small, granular sediment colonies which gradually grew to pinhead-sized flakes. Most of these were at the level of the red blood corpuscles but some were attached to the walls of the flask some distance up along the sides.

Microscopic examination revealed an immotile Gram-negative pleomorphic microbe, consisting largely of short rods, some longer threads and some coccoid bodies (Cf. Photo 1).



Photo 1 ($\times 1000$). Bocillar phase.

The colonies were transferred to a series of liquid and solid media. There was growth in ascites broth and serum broth, but not in ordinary broth. There was sparse growth on the surface of blood agar, ascites agar and solid ox serum tubes.

Growth on solid media exhibited considerable differences, both macro- and microscopically, from the above described morphology. The colonies were small and round, and, especially in serum tubes, smooth. Microscopically they consisted mostly of long, looped threads. The threads had spindle-formed swellings both at the ends and along their course. Some of the microbes were sperm-like, some resembled fungus threads with spindle-formed bodies distributed along them (Cf. Photo 2).

On the whole the microbe exhibited considerable pleomorphism with variations from tiny coccoid bacteria, coccoid bodies, to long, actinomyces-like threads. Growth was best when the medium contained both serum and red blood corpuscles. There was no fermentation of carbohydrates.

As the growth in serum tubes was readily emulgated in physiological saline, it was possible to test the patient's serum for antibodies. On Dec. 23, 1946, the patient's serum showed a positive agglutination reaction in dilution 1 : 80 with the strain, while control sera gave no positive reaction in any dilution. A new serum sample on Jan. 9, 1947 was positive in dilution 1 : 96. These antibody values agree well with previously described cases of infection with *Actinomyces muris ratti*.

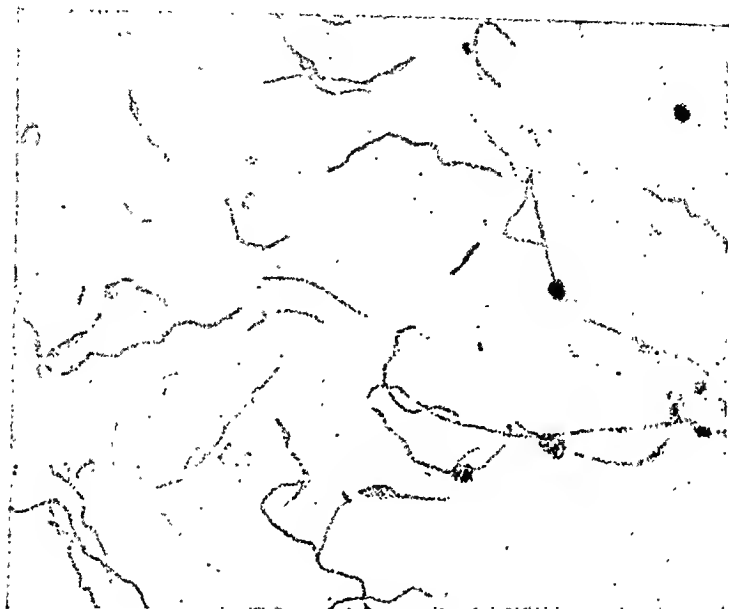


Photo 2. ($\times 1000$). Moniliform phase.

The patient's blood gave a positive reaction on Dec. 11, 1946 when inoculated in guinea-pigs and white mice. The isolated strain was later inoculated in white mice and tame rats on Jan. 9 and 11, 1947 respectively. On Jan. 23, 1947, 1 white mouse showed definitive signs of infection. It lay on its side with its hind legs drawn up and gasped for breath. It was killed and cultures were made from the blood, spleen, liver and kidneys. After a few days there was typical growth in all the cultures made in blood-serum broth tubes. The tame rats have showed no indications of infection to date.

The first description of this microbial infection after rat bite was published by *Schottmüller* in 1914 (10). He called the microbe *Streptothrix muris ratti*. He also demonstrated a similar microbe after the bite of a South African squirrel. This microbe he called *Streptothrix taraxeri cepapi*. Since then several others have found *Streptothrix muris ratti* in connection with rat bites and bites of other animals. Thus *Nixon*, 1914 (11) described a case after a ferret bite. His cultural

observations are not presented. *Blake* (12) in 1916 described a fatal case after a rat bite. Here the microbe was cultivated from blood and heart valves which were the site of an ulcerous endocarditis. *Tunncliffe* (13) the same year demonstrated a similar streptothrix in broncho-pneumonia in tame rats. *Dick and Tunncliffe* (14) in 1918 isolated a streptothrix from the blood of a patient after a weasel bite. They called the microbe *Streptothrix putorii*.

Levaditi and co-workers (15) in 1926 demonstrated the same microbe in a laboratory assistant who had been bitten by a tame rat. He called it *Streptobacillus moniliformis* which is the designation now most frequently applied to this micro-organism.

Parker and Hudson (16), 1926, use the name *Haverhillia multiformis* for a microbe which was demonstrated in connection with Haverhill fever s. *Erythema arthriticum epidemicum*. This microbe has proved to be identical with *Streptobacillus moniliformis*, so this gives a total of 4 different names for *Actinomyces muris ratti*.

Klieneberger (17) (18) has claimed in several papers that this is not one single micro-organism, but two distinct micro-organisms which live symbiotically. The one is a very fine, small organism which passes coarser, bacteria-proof filters, which *Klieneberger* calls *L. organisms*. She considers this *L. organism* to be closely related to the group of micro-organisms which are the cause of pleuropneumonia bovis. She claims that this filterable form can be isolated in pure culture from *Streptobacillus moniliformis* strains. However these are said to be unable to reproduce the filterable form.

This interpretation is emphatically contested by *Dienes* (19) who in 1939 claims that this is a variation phenomenon, a dissociation strain. This controversy is not settled at present.

Summary.

A laboratory assistant developed signs of lymphangitis and lymphadenitis shortly after being bitten by a laboratory rat. The infection improved rapidly, but 6 days after the accident the patient had chills and was sent to the hospital with high fever. On combined sulfathiazole and penicillin treatment the fever subsided at once. *Actinomyces muris ratti* s. *Streptobacillus moniliformis* was isolated from blood cultures taken before starting the treatment. The isolated microbe exhibited the same morphological and cultural properties as previously described strains. Examination of the patient's blood for antibodies 12 days after the onset of the high fever showed positive agglutination in dilution 1: 80. No case of infection with *Actinomyces muris ratti* after rat bite has previously been described in Norway, nor probably in Scandinavia.

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DISCUSSION.

S. GARD: A convenient method for demonstration of *Str. moniliformis* is intracutaneous inoculation of the sole of the foot on white mice, obtaining thus a localized infection that reminds of ectromelia with pronounced edema of the dorsal aspect of the foot, sometimes generalization with miliary liver necrosis. From the local foci, *Str. moliniformis* may easily be isolated in pure culture. Examination of apparently healthy rats, wild as well as tame, has shown the presence of *Str. moliniformis* in about 70 % of the animals, in the nasopharynx or in the middle ear.

BORDET-WASSERMANN'S REACTION CARRIED OUT WITH CARDIOLIPIN-LECITHIN-CHOLESTEROL ANTIGEN. A PRELIMINARY REPORT

By Th. M. Vogelsang.

The technique originally employed in Bordet-Wassermann's reaction has undergone great changes, and among the five factors concerned with the reaction, it is particularly the antigen which has been changed.

In 1907, and about simultaneously in three different places (Landsteiner, Müller and Potzl in Vienna, Porges and Meier in Berlin, and Levaditi and Yamanouchi in Paris) announcements were made that the original aqueous extracts of syphilitic foetal liver could be replaced by alcoholic extracts of normal organs. The antigen in Bordet-Wassermann's reaction could not therefore be an antigen in the ordinary sense; on account of its solubility in alcohol it must be of a lipoid character. In this respect the Bordet-Wassermann reaction differs from all other hitherto known serological reactions.

In 1910, Browning, Cruickshank and M'Kenzie found that the reaction became more sensitive on the addition of cholesterol to such alcoholic extracts. Since then it has become customary to use such cholesterolized antigens both for Bordet-Wassermann's reaction and the flocculation reactions. Alcoholic extracts of ox heart, to which cholesterol has been added, have in particular proved to be useful antigens.

A shortcoming of such alcoholic extracts is, however, this that they may also contain substances such as fatty acids, soaps and stearines which may have an anti-complementary action. If cholesterol is added to the raw alcoholic extract, false positive results are not rare, and the custom has therefore been adopted of purifying the alcoholic ox heart extracts by first making extractions with ether and/or acetone to remove such undesirable substances. By this means the Bordet-Wassermann reaction has become very specific and sensitive. In the overwhelming majority of cases a positive reaction is indicative of syphilis.

But even with the use of these purified ox heart extracts, we cannot prevent the occasional occurrence of false positive results. Non-specific reactions have been known for a long time to occur in certain tropical diseases. What is, however, more important in our own climate is that the same thing may happen in several more common diseases, notably in those of the respiratory tract.

Several reports of false positive reactions have appeared in Scandinavian literature. Their occurrence in large-scale investigations have been demonstrated by Krag and Lonberg (1938), by Alice Reyn (1941), and by Vogelsang (1940, 1946). Even though these non-specific positive Bordet-Wassermann reactions are not common, a further reduction of their number by more effective purification of the antigen would be a great advantage. Although for many years alcoholic tissue extracts have been used universally as an antigen for the Bordet-Wassermann reaction, we have till quite recently known very little of the chemical and physical properties of the active substance in them.

As a result of numerous investigations of the active lipid antigen constituents of these tissue extracts, there is a general belief that the substance which reacts with the syphilitic reagin in serum is a phosphatide. But it was not till 1941 that Mary C. Pangborn succeeded in isolating from ox heart a hitherto unknown serologically active phosphatide, cardiolipin. She was thus able to produce an antigen consisting of chemically pure substances.

Cardiolipin used alone as an antigen has an anti-complementary action. But mixtures of cardiolipin and purified lecithin have proved to be useful antigens whose sensitivity is considerably intensified by the addition of cholesterol.

Of late years a couple of published reports have shown that mixtures of cardiolipin, lecithin and cholesterol have been used as antigens for the Bordet-Wassermann reaction and several flocculation reactions. The changes have been rung on the technique in so far as the three components have varied in relation to each other in the different mixtures.

Harris and Portnoy (1944) were the first to undertake an intensive study of the antigen action of mixtures of cardiolipin, lecithin and cholesterol in the Bordet-Wassermann reaction carried out with Kolmer's technique. Alcoholic solutions of the three components were mixed in various proportions and diluted with saline solution in the ratios 1:75 to 1:2400 on the slow addition of the antigen to the saline solution. They achieved a satisfactory sensitivity and specificity with antigens containing 0.03% to 0.06% cardiolipin, 0.3% to 0.6% cholesterol, and about 0.05% lecithin. These antigens were suspended in saline solutions whose strength ranged from 1:150 to 1:300. No change in the serological action of such antigens could be found after storage at room temperature for one year.

Elizabeth and Frank Maltaner (1945) have tried to standardize the cardiolipin-lecithin-cholesterol antigen for the Bordet-Wassermann test. They have shown that the quantity of cholesterol to be added to a given quantity of cardiolipin in order to obtain the best results depends on the quantity of lecithin employed. They recommend the use of an antigen in which the ratio of lecithin to cardiolipin is as 5:1, and the ratio of cholesterol to lecithin is as 3.4:1. The antigen action of such mixtures was found in their tests to be independent of the antigen concentration in alcohol. For the quantity of saline solution to be added to achieve the best result was approximately proportional to the antigen concentration in alcohol. Thus the quantity of antigen used for the reaction remained approximately unchanged.

Mary C. Pangborn has kindly provided me with the cardiolipin antigen used in the comparative investigations I am about to describe. Its composition is as follows:

Cardiolipin	0.0175 %
Lecithin	0.0875 %
Cholesterol	0.3 %

It will thus be seen that the antigen contains five times more lecithin than cardiolipin, and about 3.4 times more cholesterol than lecithin.

In our experience, this antigen has no greater haemolytic action than alcohol. The same technique followed in carrying out the reaction was also adopted for the determination of the anti-complementary action with a titer of 1:4.

In the process of diluting the antigen, its quantity is measured in one cylinder, and the quantity of the saline solution in another. The saline solution is rapidly added to the antigen with which it is well mixed by pouring the fluid backwards and forwards several times from the one cylinder to the other as in the preparation of Kahn antigen.

To determine the optimum dilution, pooled syphilitic sera are titrated with various antigen dilutions. By this means the titer for the optimum dilution proved to be 1:130, 1:140. For the reaction a titer of 1:130 was used.

This new antigen was employed for Bordet-Wassermann testing of 5630 sera subjected to three parallel tests used as a matter of routine at Gade's Institute: Bordet-Wassermann with an extract of ox heart to which 1 % cholesterol had been added, the Kahn standard test, and Meinicke's »Klärungs« test. For the ordinary Bordet-Wassermann test as well as the reaction carried out with cardiolipin antigen, Eagle's method was used with 0.2 ml. of each of the five factors and a total quantity of fluid 1. ml.

The 5630 sera examined were classed in two groups according as anamnestic or clinical evidence of syphilis was present or lacking.

Table 1.

Sera from	Number of sera	Identical results			Conflicting		
		Number of sera	+	÷	Number of sera	B-W.	C. I. c.
Untreated Syphilis	205	198	178	20	7		7
Treated Syphilis ..	378	338	127	211	40	8	32
Latent Syphilis ...	117	108	51	57	9	3	6
Syphilis	+ 700	644	356	288	56	11	45
Syphilis	÷ 4930	4895	30	4865	35	29	6

C. I. c. — Cardiolipin-lecithin-cholesterol antigen.

Table 1 compares the Bordet-Wassermann reactions according as they were carried out with extract of ox heart or cardiolipin-lecithin-cholesterol antigen. The 700 syphilitic sera were classified in different groups according as they were examined with a view to the diagnosis of syphilis and before specific treatment had been instituted, or they were examined in the course of such treatment or after its completion for the purpose of supervision during the following four years. The sera from patients who had not received specific treatment or had not presented clinical evidence of syphilis during the last four years before the examination were included under the heading: latent syphilis. On the whole there was quite good agreement over the figures concerning patients with untreated and latent syphilis, but among the 378 sera from treated cases there were as many as 40, or more than 10 %, giving conflicting results. In eight of these cases the reaction was positive only with extract of ox heart, and in as many as 32 cases, it was positive only with the cardiolipin antigen.

Among the 700 sera from persons known to be syphilitic there were 644, or 92 %, giving concordant results. About one-fifth of the remaining 56 sera gave a positive reaction only with extract of ox heart, while about four-fifths did so with the cardiolipin antigen.

There was no evidence, clinical or anamnestic, of syphilis connected with 4930 sera about 0.7 % of which gave conflicting results. Here we find the situation reversed, for about four-fifths of these 35 sera gave a positive reaction only to extract of ox heart and only about one-fifth to cardiolipin antigen.

Further to form an opinion of the usefulness of the cardiolipin antigen, it was employed not only for our routine Bordet-Wassermann testing, but also for a comparison with the two flocculation tests which are carried out on the current material in the Institute (see table 2). Here, too, it will be seen that the greatest discrepancies are to be found among the treated cases of syphilis. The results were conflicting in 22 % of the 700 syphilitic sera examined. When one of the tests failed us, this was the case in only a couple of sera tested with cardiolipin antigen. Of the sera giving a positive reaction to two

Table II.

Sera from	Number of sera	Identical results			Conflicting results			
		Number of sera	+	÷	Number of sera	Number of positive results		
						3	2	1
Untreated Syphilis..	205	184	169	15	21	11(11)	8 (5)	2
Treated Syphilis ..	378	273	93	175	105	36(33)	42(24)	27(4)
Latent Syphilis	117	87	43	44	30	13(12)	12 (2)	5
Syphilis	+700	544	310	234	156	60(56)	62(31)	34(4)
Syphilis	÷4930	4824	6	4818	106	14(11)	55(19)	37

() — Number of positive results with C. l. c. antigen.

tests, half their number gave a positive reaction to the cardiolipin antigen, whereas most of the sera giving a positive reaction to only one test were negative to the cardiolipin antigen.

With regard to the sera from persons with no evidence of syphilis, and giving a positive reaction to several of the other tests, the reaction to the cardiolipin antigen was usually positive. None of the 37 sera giving a positive reaction only to one test did so to the cardiolipin test.

In Western Norway, with its great distances and to some extent still faulty modes of transport, samples of blood may take several days to reach the Institute which not infrequently receives haemolyzed and decomposed sera for this reason. The sera which are not too obviously damaged are included in the routine examinations, and in the period under review as many as 41 sera had to be excluded from this analysis because they showed marked anti-complementary action.

Among the 700 sera from persons with evidence of syphilis, we find the following:

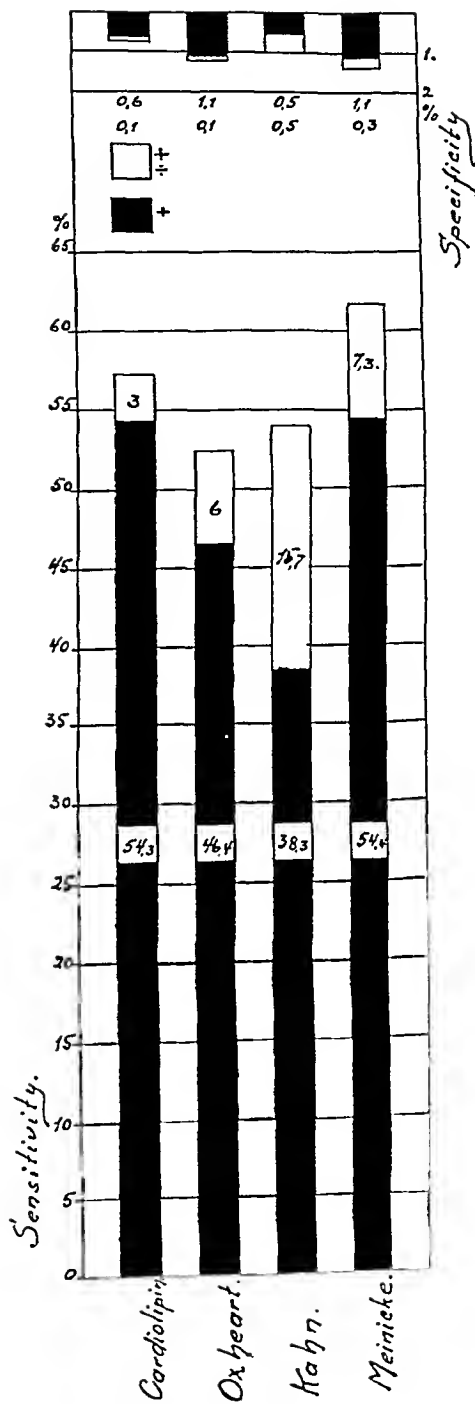
401 positive to cardiolipin antigen.
 367 " " extract of ox heart.
 378 " " Kahn.
 432 " " Meinicke.

Among the 4930 sera from persons without evidence of syphilis, we find the following:

36 positive to cardiolipin antigen.
 59 " " extract of ox heart.
 51 " " Kahn.
 67 " " Meinicke.

The sensitivity and specificity of each test is graphically demonstrated in the chart.

It will be seen that, in syphilitic sera, the Bordet-Wassermann reaction with cardiolipin was more often positive than with extract of ox heart and was also more sensitive than Kahn, but less so than Meinicke.



As for the specificity of the Bordet-Wassermann reaction with cardiolipin, it will be seen that in my material it gave the smallest number of false positive results. Among the sera unaccompanied by any evidence of syphilis, there were 1487 sent for the examination of pregnant women in connexion with recent legislation in Norway; 585 were from healthy persons willing to serve as blood donors, or requiring health certificates, etc. Among these 2072 sera were five giving positive Bordet-Wassermann reactions to both extract of ox heart and cardiolipin. There were also four giving a positive reaction only to extract of ox heart. Among the other persons without evidence of syphilis were 271 who suffered from diseases of the respiratory tract. Four of them gave a positive reaction to both cardiolipin and extract of ox heart, and four others of them did so only to the latter. Among my cases there was one of malaria and two of infectious mononucleosis; all three gave a positive reaction to extract of ox heart, but a negative reaction to cardiolipin.

Although the Bordet-Wassermann test carried out with the cardiolipin antigen has proved to be very specific, it would seem that even with this antigen we cannot altogether escape false positive reactions. Cardiolipin is presumably one of the antigen lipoids to be found in alcoholic extracts of ox heart. When a pooled positive serum is absorbed with extract of ox heart, the reaction becomes negative not only with this extract, but also with cardiolipin antigen. But it would seem that an alcoholic extract of ox heart also contains other lipoids which may possess an antigen action and may occasionally give rise to false positive results.

Far more comprehensive investigations than those recorded here are necessary for a better knowledge of the properties of cardiolipin antigen, and the present report is therefore only preliminary. My investigations seem, however, to confirm the impression that, with the aid of chemically pure substances, it is possible to produce an antigen which is satisfactorily sensitive and so reliably specific that it can be employed in the complement fixation test for syphilis.

It is well known that extracts of ox heart are apt to vary somewhat when each serological laboratory makes its own preparations. The cardiolipin-lecithin-cholesterol antigen possesses this advantage that it is easily standardized and can therefore help us to achieve more uniform results.

Summary.

- (1) The Bordet-Wassermann reaction carried out with the cardiolipin-lecithin-cholesterol antigen has been found to be as sensitive as when carried out with the ordinary cholesterinized extract of ox heart.
- (2) The Bordet-Wassermann reaction carried out with the cardiolipin-lecithin-cholesterol antigen has given fewer false positive reactions than with the ordinary cholesterinized extract of ox heart.

(3) The cardiolipin-lecithin-cholesterol antigen possesses this advantage that it is easily standardized, and its general use in different laboratories should therefore help to give more uniform results.

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COMPLEMENT FIXATION IN HEPATITIS WITH SOME LIPOIDS FROM LIVER-TISSUE

By M. Bjørneboe, P. Krag and F. Lundquist.

In earlier experiments*) it was demonstrated, that the complement-fixating effect in liver-suspensions resisted drying at 40° and subsequent storing at room temperature.

Knowing that liver-suspensions react with all WR positive sera, while WR-antigen reacts only with a small number of hepatitis positive sera, it seems probable, that the liver-suspensions include two antigenic fractions. We have studied several lipid fractions from normal human liver, and have found, that both substances soluble in ether and substances insoluble in ether but soluble in alcohol may react with hepatitis sera — therefore the following fractions were prepared:

Dried liver powder was extracted with hot ether — then Sphingomyelin precipitated during cooling — Cephalin precipitated by alcohol and Lecithin taken as the soluble rest. From each fraction is prepared a suspension in saline containing the lipoids in the same concentration as in the untreated liversuspension.

In the complement fixation Cephalin showed no reaction with hepatitis sera. The other preparations gave promising results tested with sera that in previous experiments had shown reactions with liver suspensions:

Mean value of strength in fixation tests.

	Number of sera tested	Lecithin	Sphingo- myelin	Non-treated liver
Hepatitis sera	2	0	9	9
Syphilis and Hep. sera ..	3	6	3	4
Syphilis sera	3	5	1	2
No liver disease	1	not tested	0	8

*) Acta path. XXIV. 352. 1947.

New amounts of sphingomyelin and lecithin were prepared from the same liver-powder — the two fractions were purified by repeated precipitations (sphingomyelin precipitated from methanol with Ammonium Reineckate. These two new preparations did not show the above demonstrated difference in complement fixation power — in fact they resembled each other highly; this suggests that the wanted liver-fraction has not been isolated or consists of an unknown mixture of lipoids.

Both liver-suspensions and sphingomyelin-suspensions show increased reactivity, when a small quantity of cholesterol is added.

Investigations on these lines are continued in our laboratory.

NO DISCUSSION

COMBINED DIPHTHERIA AND TETANUS IMMUNIZATION

By *Inga Scheibel & Knud Bojlén.*

Hitherto the interest in active immunization against tetanus has been rather very slight in Denmark and, it is our impression that the same applies to the other Scandinavian countries. For a long while, we think physicians were inclined to rest satisfied with the serum prophylaxis — which naturally meant an enormous advance in the fight against tetanus. After the adoption of this measure during the previous world war the tetanus morbidity among the wounded soldiers fell from 9 % to 1.4 %.

Now and then, however, in spite of thorough prophylaxis, instances of tetanus make their appearance. In cases with a long incubation period or in cases of slowly healing wounds, in which a toxin-producing focus possibly still may persist, there is a risk that the antitoxin given to the patients may have become eliminated at a point of time when the production of toxin still may take place. Besides, now and then a case of tetanus may set in after such a slight injury that the patient had not found it necessary to seek medical advice.

Considering, moreover, that the prophylactic serum treatment not infrequently gives a moderate or more severe degree of serum sickness — the incidence is given as being up to 40 % — and that through the injection of serum we run a risk of sensitizing several of the patients to horse serum — which naturally will be rather inconvenient if later on in life some form of serum therapy is indicated — it may reasonably be said that passive immunization against tetanus is no ideal prophylaxis.

To us, however, this requirement seems to be met almost completely by the other possible way of protecting against tetanus: the active immunization.

Of course, it may be said that in peace time tetanus plays only such a modest rôle that it would be unreasonable to inaccomodate the

public with an additional vaccination. Yet, according to the data furnished by Perdrup, nearly 40 cases of tetanus occur every year in this country, 15 of them with a fatal outcome. If we reckon, however, that a majority of these cases — and presumably all the fatal — might have been avoided by active immunization, it seems to us to be highly worth while again to try to awaken an interest in this form of prophylaxis.

The principle of the immunization is the same as in immunization against diphtheria, namely: antibody production after injection of tetanus toxoid, i. e., tetanus toxin which through a formalin-heat treatment has lost every trace of toxicity but preserved its antigenic capacity.

The fundamental work in the field of immunization in tetanus was carried out by Ramon & Zoeller. In 1928, in the French cavalry an extensive experiment was carried out with a view to this point, and the results thus obtained appeared very convincing: In spite of serum prophylaxis, cases of tetanus appeared among the non-immunized horses at the usual rate of frequency (about 4 ‰), whereas no instance whatever turned up among 35,000 horses which had been given 3 anatoxin injections. Ten years later, some of these horses were re-examined, and they were found still to be fully protected against a massive infection with tetanus spores. Since 1936, immunization against tetanus has been obligatory for the soldiers in the French army. In 1939 both the British army and R. A. F. introduced immunization against tetanus. Like all other vaccination in England, this was a voluntary measure, and yet 90—100 % applied for the immunization. In 1942 the army of U. S. A. adopted tetanus immunization as a compulsory measure.

In Denmark the first tetanus immunizations were performed by Schmidt, Madsen & Ahrend Larsen in 1930 and 1935 when a part of the personnel of the State Serum Institute was immunized, with excellent result. In spite of the encouraging experiments in other countries as well as here, active immunization against tetanus has been employed hitherto only to a very slight extent. It was our hope, therefore, that perhaps it might be practicable to make use of the strong foothold which the diphtheria immunization gradually has gained in the Danish population and, so to speak, slip tetanus immunization into general practice by preparation of a prophylactic containing both diphtheria and tetanus antigen, so that an effective immunity against both diseases might be obtained simultaneously without any increase in dose or number of injections.

In other countries, especially in France and America, combined immunization has been employed to a large extent. Thus, in France the immunization with diphtheria-tetanus prophylactic has been compulsory for children of 1—14 years since 1940. The prophylactics used, however, are prepared in rather different ways, and hence it was necessary more thoroughly to examine the Danish mixed prophylactic

in order to ensure that it was fully effective with regard to both antigens.

It will be appropriate quite briefly to mention the very preparation of the prophylactic and the first orienting experiments with it that were carried out on guinea-pigs. Both the diphtheria toxin and the tetanus toxin are submitted to a thorough purification by filtration through collodion membranes with pores of such a size that the main part of the unspecific low-molecular substances pass through the membrane while the specific substance is retained. The purified toxin is detoxicated with small amounts of formalin at 32°, and then the excess of formalin is removed by a repeated ultrafiltration. Now the preparations have become so poor in nitrogen that in dilutions of 50 Lf units diphtheria anatoxin and 25 Lf units tetanus anatoxin they are completely adsorbable on 10 vol. % aluminium hydroxide — that is, the same amount as we employ in the unmixed diphtheria prophylactic.

In experiments on guinea pigs with this mixed prophylactic it was found to induce an excellent antibody production against both antigens. Still, a certain degree of antigenic antagonism could be demonstrated, as higher titers were obtained by immunization with the unmixed prophylactics separately. But this inhibition of the antibody production was of such a low magnitude that it could hardly be imagined to play any practical rôle. Indeed this view was later confirmed by the outcome of the immunization results on human subjects which now will be presented.

This investigation comprises altogether 34 persons, 20 of whom were students, 14 inmates in an asylum.

The vaccine contained per cc.: 25 Lf units diphtheria anatoxin and 25 Lf units tetanus anatoxin adsorbed on to 10 % $\text{Al}(\text{OH})_3$. The dosage was the same as usually employed in Denmark: 2 injections of 1 cc. at an interval of 4 weeks and a 3' injection about 1 year later. The antitoxin content of the serum was examined before the first injection, 1—3 months after the 2' injection, prior to the 3' injection, 9 days after the 3' injection and, in some cases, also 2½—3 years after the commencement of the immunization.

Unfortunately it has not been possible quite consistently to carry through these examinations, which have been going on for some years, so that in the calculation of the mean titers it is only in some degree that the various calculations cover precisely the same persons. The antitoxin curves for the individual subjects proved to take a very uniform course, and the titers obtained for the persons examined at different points of time were distributed fairly uniformly with regard to low and high values. We therefore found it justifiable anyhow to employ the usual mean value calculation as a surveyable expression for the course of the immunization. First we looked into the question whether the age of the subject has played any part with regard to the antibody production.

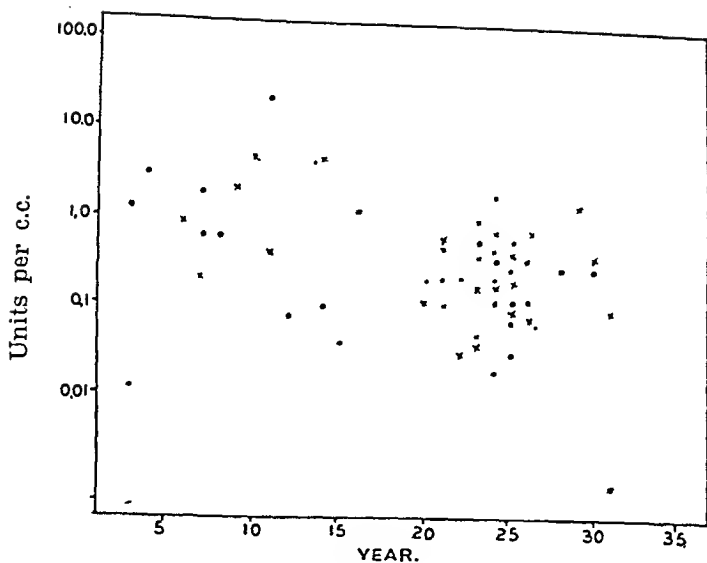


Fig. 1.

In the graph presented in Fig. 1, the age of the subject is plotted along the axis of abscissas while the antitoxin production in units (A. U.) after the 2' injection is plotted logarithmically on the ordinate. x gives the diphtheria titers, while the points signify the tetanus titers. It will be noticed that at no age is there any accumulation of high or low titers. So, in the further treatment of this material we have left the age difference of the subject out of consideration.

Table 1 illustrates the diphtheria antitoxin production. In this tabulation we have entered only the subjects who prior to the immunization contained no measurable amounts of natural antitoxin, because even though a naturally immune organism contains but very small amounts of antitoxin it will respond to an injection of vaccine with a far stronger antitoxin production than does a non-immune organism (Glenny & Südmersen).

For the sake of comparison we have also recorded the titers obtained after diphtheria immunization of a group of persons picked out at random in Copenhagen who had been immunized with a corresponding diphtheria prophylactic without any admixture of tetanus antigen (Table 1 b).

As will be noticed from Table 1 a and b, the antibody formation after the two immunizations takes a very uniform course. Neither the immediate response nor the stability of the immunity show any greater differences than may be due to experimental errors. The condition that has to be met by the prophylactic — that it protects effectively as many subjects as possible — is met just as well on employment of the mixed prophylactic as when the diphtheria prophylactic is employed alone. 9 days after the 3' injection of the mixed prophylactic

Table 4.
Diphtheria Antitoxin Titer.

a) Immunization with combined Diphtheria-tetanus prophylactic.

	3-12 weeks after 2' inj.	12-18 months after 2' inj.	9 days after 3' inj.
A. U. per c.c. (Geom. mean).	0.42 (27)	0.091 (22)	5.64 (18)
≤ 0.01 A. U. per c.c.	0	4.5 %	0

b) Immunization with Diphtheria prophylactic.

	3 weeks after 2' inj.	One year later
A. U. per c.c. (Geom. mean).	0.51 (38)	0.065 (25)
≤ 0.01 A. U. per c.c.	5.1 %	8.0 %

c) Immunization with combined Diphtheria-tetanus prophylactic.

	18-21 months 3' inj.	2 days after 4' inj.	4 days after 4' inj.	6 days after 4' inj.
A. U. per c.c. (Geom. mean).	0.32 (9)	0.36 (9)	0.60 (9)	6.92 (9)
≤ 0.01 A. U. per c.c.	0	0	0	0

d) Immunization with Diphtheria prophylactic.

	2½ years after 3' inj.	7 days after 4' inj.
A. U. per c.c. (Geom. mean).	0.23 (12)	3.46
≤ 0.01 A. U. per c.c.	0	0

the diphtheria antitoxin titer has increased to 5.64 units i. e., more than 60 times. For this injection, unfortunately, we have no suitable control material. But, we think, this rise may be said to be of such a magnitude as to be convincing in itself.

1½—2 years after the 3' injection we had occasion to take samples of blood from 9 of the vaccinated subjects, who at the same time were given an additional injection (4' injection). As will be noticed from table 1 c, all these subjects still showed an antitoxin content over 0.01 unit per cc. before injection. Additional samples of blood were taken 2, 4 and 6 days after the injection in order to see how soon the antibody production would be set a-going in a sensitized organism.

For comparison we have recorded the titration results after immunization with diphtheria prophylactic alone performed in 1941 on inmates in the same institution as those examined in the present work (Table 1 d).

Thus, an additional injection (injection de rappel) — the significance of which to the stability of the protection against diphtheria has been emphasized especially by Ramon & Zocler — gives also a

rapid and pronounced increase in antitoxin on employment of the combined prophylactic. This rise commences between 2 and 6 days after the injection. The antitoxin determination on the 4' day shows a slight rise, it is true, but the few observations in this group do not allow of any definite conclusion in this respect. In contrast hereto between the 4' and 6' days the antibody production sets in almost explosively, and it reaches the same level as after immunization with diphtheria prophylactic alone.

So, as far as protection against diphtheria is concerned, there appears to be nothing that may be taken as an objection to the employment of a combined prophylactic — something that is quite in keeping with the outcome of American and French investigations into this problem.

In judging of the result of the tetanus immunization we meet with no complication of the problem from the occurrence of natural immunity, as natural tetanus antitoxin appears not to develop in man. It is true that Tenbroeck & Bauer in 1923 reported that in a group of Chinese they had found natural tetanus antitoxin, but no such observation has ever been reported since. Among others, Ericsson and collaborators (1944) examined a fairly large number of recruits in a Stockholm regiment without finding any instances of natural antitoxin. Nor has it been possible to demonstrate the presence of antitoxin in persons who have survived a tetanus infection.

The minimum amount of tetanus antitoxin to be required for protection against tetanus is difficult to estimate. In the literature statements on this point vary from 0.3 units per c.c. to about 0.001 units per c.c. In prophylactic serum therapy the usual dose is 3000 units injected intramuscularly; and according to Madsen and Wolter & Dehmel this gives a maximal titer of 0.2—0.5 unit per cc. on about the 3' day. After this, the titer keeps falling daily, and after 2—3 weeks it is no longer measurable. In their studies, Ericsson *et al.* found less than a total content of 250 units — or less than 0.1 unit per cc. — in 77 % of the subjects 8 days after the injection of 3000 units. According to Sacquépée & Jude the antitoxin titer falls even more rapidly in patients who have received horse serum before, so that here the period of protection cannot be reckoned to last longer than about one week.

According to the experiments reported by d'Antona & Valensin and Bergey, Brown & Etris, actively immunized guinea-pigs appear to be better protected than passively immunized animals showing the same antitoxin content at the time of the infection. Actively immunized guinea-pigs were able to stand the injection of 200 x M. L. D. of a tetanus toxin without having symptoms of tetanus when their serum contained about 0.02 unit per cc., whereas passively immunized guinea-pigs containing about 20 times more antitoxin (about 0.4 unit per cc.) died or had very severe tetanus from injection of the same amount of

toxin. Wolter & Dehmel injected on themselves subcutaneously a dose of tetanus toxin sufficient to kill 300 mice two and a half years after these authors had been actively immunized with 3 injections of tetanus prophylactic. At this point of time the authors showed an antitoxin content of about 0.01 unit per cc., and both were able to stand the injection without any inconvenience whatever.

On the basis of these various experiments we have chosen the antitoxin content of 0.02 unit per cc. as the minimum requirement. The same value has been adopted by several American investigators, while Eriesson and collaborators suggest 0.01 unit.

Table 2.
Tetanus Antitoxin Titer.

a) *Immunization with combined Diphtheria-tetanus prophylactic.*

	3-12 weeks after 2' inj.	12-18 months after 2' inj.	9 days after 3' inj.
A. U. per c.c. (Geom. mean).	0.19 (31)	0.012 (29)	13.2 (25)
≤ 0.02 A. U. per c.c.	6.4 %	55.2 %	0

b) *Immunization with Tetanus prophylactic.*

	6 weeks after 2' inj.	1 year after 2' inj.	2-3 weeks after 3' inj.
A. U. per c.c. (Geom. mean).	0.11 (36)	0.013 (35)	0.59 (36)
≤ 0.02 A. U. per c.c.	27.8 %	77.2 %	0

c) *Immunization with combined Diphtheria-tetanus prophylactic.*

	1½-2 years after 3' inj.	2 days after 4' inj.	4 days after 4' inj.	6 days after 4' inj.
A. U. per c.c. (Geom. mean).	0.23 (15)	0.28 (15)	0.24 (15)	2.88 (15)
≤ 0.02 A. U. per c.c.	13.4 %	13.4 %	6.7 %	0

We have had no opportunity ourselves to perform control experiments with tetanus prophylactic alone. The control values given in Table 2 b are taken from the investigations reported by Wolter & Dehmel, who employed a tetanus prophylactic of similar composition and potency as the Danish one.

Also the tetanus antitoxin production appears not to be influenced by the fact that a prophylactic combined with diphtheria antigen was employed. The mean values are practically alike except after the 3' injection (last column), where it looks as if the combined prophylactic has produced the stronger antibody formation. After the 2' injection, 2 out of 31 subjects (i. e. 6.4 %) were found to show an antitoxin titer lower than 0.02 unit per cc.; from 12 to 18 months later the titer had fallen below 0.02 unit in 16 out of 29 subjects=55.2 %. The correspond-

ing figures after immunization with tetanus prophylactic alone are respectively 27.8 and 77.2 %. Thus a combined prophylactic has brought about an antitoxin titer of ≥ 0.02 unit per cc. in at any rate just as many subjects as had the tetanus prophylactic alone. After 3' injection a very pronounced increase in mean titres is seen in both groups. The difference between them may find its explanation in the difference of interval between injection and blood tests.

Whether it really may be safe not to give immunized injured patients any tetanus serum but simply a repeated injection of prophylactic, will depend on the length of the incubation period and of the rapidity with which the antibody production sets in after repeated injection of prophylactic. Therefore we have looked into the effect of an injection of prophylactic given $1\frac{1}{2}$ —2 years after the 3' injection, that is, corresponding to what will happen in an immunized person who suffers a traumatic injury and then is given an injection of prophylactic instead of serum (Table 2 c).

It will be noticed that only in 13.4 % of the present subjects does the serum contain less than 0.02 units per cc. as long as $1\frac{1}{2}$ —2 years after 3' injection, whereas the antitoxin titer after the 2' injection fell below this value after 1— $1\frac{1}{2}$ years in no less than 55.2 % of the subjects. This illustrates once more the importance of the 3' injection. In 6 days after the injection there was a pronounced rise in the titer in all the vaccinated subjects but one who, on the other hand, had shown an unusually high titer (5 A. U. per cc.) prior to the last injection.

The incubation period for tetanus is stated to be 6—14 days. In rare cases it may be a little shorter, and not altogether infrequently it is a little longer.

Thus we have found that before the end of the incubation period, the antitoxin production in a fully immunized individual in response to an additional injection of prophylactic will be increasing strongly at a point of time when toxin formation still is too slight to manifest itself. From 4 to 6 days after the injection and in the following days or weeks the antitoxin titer will be rising steadily, and then it will fall slowly — in contrast to the passive immunity which will have disappeared after a couple of weeks, occasionally even as early as after one week. So our findings may be taken to indicate that it will be safe to replace serum injection with an injection of prophylactic that should be given within 24 hours after the injury.

This procedure has been employed in the American and French army and air forces in the last world war. In the British army and in R. A. F. both serum and an injection of prophylactic have been given to all the wounded, while the troops from the colonies every year were given an injection of prophylactic that was repeated if they were wounded.

As yet the reports from the war on this point are rather scanty

and accidental. Still, like our experiments, they indicate that the injection of serum may be replaced with an injection of prophylactic. When the final reports are published, no doubt, there will be a possibility of getting this question elucidated so thoroughly that it may be settled conclusively.

DISCUSSION

VOGELSANG, TH.: I have been greatly interested in the paper read by Dr. Scheibel. It has been discussed at great length, indeed, whether an effective immunization may be obtained by combining the diphtheria vaccine with other vaccines. From the investigations reported by Inga Scheibel and Knud Bojlén it is evident that the immunizing effect obtained by simultaneous injection of diphtheria-anatoxin and tetanus-anatoxin is just as good as the effect from their separate injection at different points of time.

The primary aim must be to get the population immunized against diphtheria. It would be preferable to vaccinate the children with two injections in the second half of the first year of life and give the third injection one year later, and then — in order to obtain a long-lasting immunizing effect — a new injection at the beginning of school age, and the final injection when the children leave school. Hereby it should be possible gradually to get the entire population immunized against diphtheria.

After the examinations performed, in this connection, the question is to be ventilated whether the children also should be given tetanus vaccine simultaneously with the diphtheria vaccine. To settle this question, I hope that Dr. Scheibel and Dr. Bojlén will continue their studies on a larger material.

When it comes to vaccination of recruits, on the other hand, I think that the vaccination against diphtheria alone ought to be replaced as soon as possible with the combined diphtheria-tetanus vaccination.

SCHEIBEL, INGA: We have not been able to demonstrate any better antibody production after vaccination with combined vaccine, merely the same as fixed. When we advise against simultaneous vaccination against diphtheria, and for instance, typhoid fever, it is not so much with a view to the antibody production as to the implied risk of complications.

It is our intention to try to carry through the combined vaccination on children as a matter of routine, and to suggest tetanus vaccination of the recruits who already have been vaccinated against diphtheria, while combining the two vaccinations for the remainder.

In the transitional period, I think, in many cases we will have to continue with the passive prophylaxis.

IN VITRO METHOD FOR TESTING THE TOXIN-PRODUCING CAPACITY OF DIPHTHERIA BACTERIA

By *Örjan Ouchterlony.*

There exist at present several methods for testing the toxin-producing capacity of diphtheria bacteria and some are very well known and minutely elaborated. Roux, Ehrlich, Römer, Ramon and Claus Jensen are all names connected with this field of investigation, where the experiences also many a time have opened a way to investigations of other kinds of toxin-producing bacteria. Much theory and speculation concerning antigen-antibody reactions in general and toxin-antitoxin reactions in particular is also based on observations of diphtheria toxin-antitoxin.

The theoretical background to the method for toxicity testing, of which there will be given an account below, will only be quite summarily treated, *inter alia* for want of space, but is to be published in a separate paper.

The present account is only intended to demonstrate how an observed manifestation of diphtheria toxin-antitoxin binding occurring in semisolid media, has been arranged to be of best use to the diphtheria diagnostic laboratory work.

First there will be given a brief account of some explanatory model tests. These tests have been made in Petri dishes with serum agar (1 % agar, 50 % serum). In test 1 two so-called penicillin cups were placed on the surface of the medium, about 3 cm from each other. Diphtheria toxin (27 FIU/ml) was poured into one of the cups and antitoxin (45 FIU/ml) into the other. The plate was then incubated at 37° C. After two days a thin streak could be observed between the cups and it grew sideways little by little. Beside the primary distinct streak a couple of adjacent streaks, somewhat less distinct, were also observed. — Test 2 was arranged in the same way, but here the toxin was first heated (70° C — ½ hour). A distinct primary streak as in test 1 was however not to be seen in this test.

In test 3 diphtheria immune serum to a content of 10 FIU/ml medium was added to the serum agar in the plate. Three cups placed on the medium were filled with diphtheria toxin of the concentrations 25, 20 and 15 FIU/ml, after which the plate was incubated at 37°. After 48 hours a halo formation, whose diameter was larger the lower the toxin strength in the cup had been, could be observed around each cup. On continued observation it could be seen that the diameter of the halo increased and that multiple halo phenomena appeared.

In test 4 a series of immune serum plates with falling quantity of antitoxin 10—5—2.5—1.25 FIU/ml medium was made. On each plate there was placed a cup containing toxin of strength 25 FIU/ml and the plates were incubated at 37° C. After one or two days there appeared around the cups a halo formation. The larger the diameter of the halo, the lower the content of immune serum of the plate. The first halo observed was formed in the plates with a high content of immune serum.

In test 5 a smearing of the strongly toxin-producing strain PW 8 was made on an immune serum plate (5 FIU/ml medium), which was then incubated at 37°. After 24 hours there appeared around the growing strain a halo, which increased in strength during the following days.

In test 6 some diphtheria strains, which had shown themselves toxic or atoxic in guinea-pig tests, were smeared on a plate as in test 5. After some days there could be seen halo formations around the smearings of the toxic strains, but not around the smearings of the atoxic strains.

In test 7 finally, a transversal trench was cut out of a serum agar plate and immune serum agar was poured into this trench. Across the plate, at right angles to the trench, the strain PW 8 and some diphtheria strains, toxic and non-toxic to guinea-pigs, were smeared. The plate was then incubated at 37°. Between 24 and 96 hours afterwards there could be observed moustache-like streaks, going out from the bacterial growth, in the angle between the trench and the growth (Fig. 1). the first strain to produce this streak was PW 8. The primary distinct streaks originating from this strain and the toxic strains gradually turned off to the corresponding streak of the adjacent strain and finally joined it. Weaker streaks, appearing somewhat later, were observed, above all from the atoxic strains. These streaks did not interfere with the primary more distinct streaks but crossed them, apparently without being affected.

The observations described in the model tests above illustrate the author's working hypothesis, on which the following practically used *in vitro* method has been based.

The following observations and presumptions have thus been made. If an antigen and the corresponding precipitating or flocculating anti-

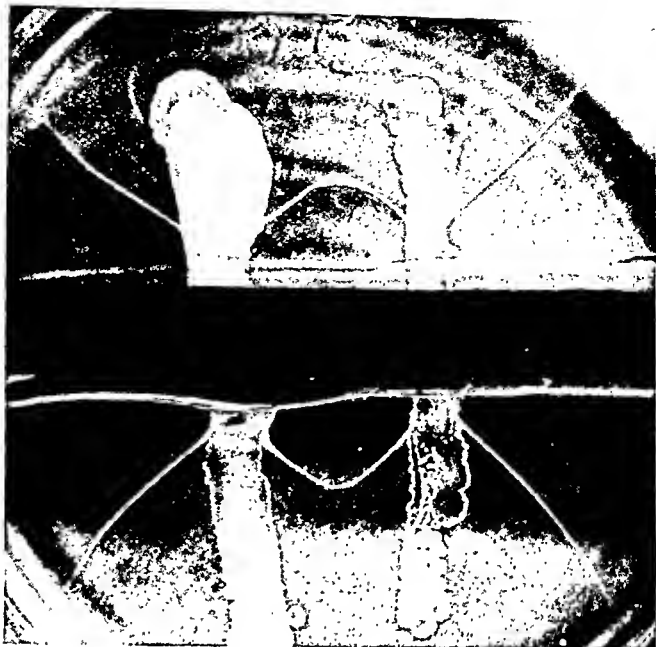


Fig. 1.

Serum agar plate with a trench containing immune serum agar as in model test 7. Two strokes of the strain PW 8 demonstrating interference phenomena.

body diffuse towards each other in an indifferent gel of suitable consistency, e. g. gelatin or agar, there will appear, under certain circumstances, a streak- or band-like precipitation in the gel between the two diffusing components. The reaction is probably of the same nature as that between an antigen and antibody. The position of this band of precipitation in relation to the two diffusion centres depends *inter alia* upon the initial concentration and the diffusion velocity of the two reacting substances. A similar reaction is obtained if antigens are allowed to diffuse in gels with a suitable constant content of antibodies or vice versa. In these cases the reaction appears at varying distances from the diffusion centre, depending partly on the concentration conditions, the diffusion velocity and the time elapsed before the reaction becomes visible.

If different antigens and antibodies have been mixed, multiple reactions appear and the different systems react independently of one another. In these cases it is possible through a special proceeding to obtain interference and crossing phenomena, which may be of help in the qualitative analysis (test 7). The diffusion method can also be arranged so as to allow quantitative estimations within certain limits (tests 3 and 4).

In the practical tests of the *in vitro* method 545 strains of *Corynebacterium diphtheriae* were examined parallelly to guinea-pig tests

(subcutaneous inoculation from 48 hours serum broth pure cultures). These strains were fresh pure cultures from throat and nose swabs sent in for bacteriological examination to the Diphtheria Department of the Laboratory. As selective primary substrate Clauberg-Herrmann's so-called indicator plates (tellurite-dextrose-waterblue) were used.

In the *in vitro* tests all strains were examined by making shokes on a series of serum agar plates with a falling content of diphtheria immune serum. The plates were read after 48 and 96 hours and then after 8 and 16 days. They were incubated at 37° during the first 96 hours but then kept at room temperature. The appearance of a specific halo around the bacterial growth was registered as a positive reaction. Besides the strains to be tested the strain PW 8 was inoculated on all plates as a control.

The investigation material was divided into two groups. In the first, 237 strains were examined by using a series of serum agar plates with an antitoxin content of 12.5—10.0—7.5—5.0—2.5 and 1.3 FIU/ml medium respectively. In the other group 308 strains were examined by using a series of plates with an antitoxin content of 40—20—10—5—2.5 FIU/ml medium respectively. Moreover these plates contained potassium tellurite to 0.16 %.

In the first group the time after which a positive reaction for the 126 toxic strains could be visible was 48 hours in 92 % and 96 hours in 8 %. No further positive reactions could be observed on continuing the control for two weeks. The highest antitoxin concentration at which a positive reaction could be observed was 12.5 FIU/ml in 31.7 %, 10.0 in 29.4 %, 7.5 in 21.4 %, 5.0 in 10.3 %, 2.5 in 6.3 % and 1.3 in 0.8 %. None of the strains had 0.63 FIU/ml as its highest reaction value. Calculated on the antitoxin concentrations 10—5—2.5—1.25 FIU/ml the majority of the strains showed a reaction in two or three plates with an adjacent content of immune serum. More exactly, in 14 % the zone width was one, in 65 % two and in 22 % three consecutive concentrations.

In the other investigation group the time after which a positive reaction might be seen, was 48 hours in 90 % and 96 hours in 10 %. No further positive reactions could then be observed. The highest content of antitoxin, which caused a positive reaction, was in this group 40 FIU/ml in 1.1 %, 20 in 52.1 %, 10 in 36.0 % and 5 in 10.8 %. The majority of the strains gave a positive reaction in two or more consecutive immune serum concentrations.

A summary of the results of the comparative *in vivo* and *in vitro* tests is made in tables 1, 2 and 3. A lack of conformity between the two methods in the primary testing appeared in 8.5 % in the first investigation group, and in 6.2 % in the second group. The results of repeated comparative tests of these strains are demonstrated in table 2. It is here shown that with certain strains, which were toxic when examined with one of the methods, the toxicity could not be

verified as the investigation continued. This result and the initial discrepancy between the two methods might to a certain extent be explained in the following way. During the course of the investigations the tested material may have changed so that certain strains have lost their toxinproducing capacity or that on continued cultivation only atoxic variations have been transferred.

Table 1.
Results of the comparative in vitro and in vivo tests.

Number of strains tot. 545	Group 1. Plates without tellurite 237	Group 2. Plates with tellurite 308
Results	%	%
G — P —	43,9	34,4
G + P +	47,6	59,4
	91,5	93,8
G + P —	5,1	2,0
G — P +	3,4	4,2
	8,5	6,2

G. guinea-pig test
P. plate test

Table 2.
Results of repeated comparative tests of the strains showing lack of conformity in the primary examination.

Group 1.
Tot. 237 strains
20 strains with discrepancy
in primary tests.

Prim. test	Repeat. test	
	G+ P+	G- P-
G+ P- 12	7	5
G- P+ 8	8	—

G. guinea-pig test
P. plate test

Group 2.
Tot. 308 strains
19 strains with discrepancy
in primary tests.

Prim. test.	Repeat. test	
	G+ P+	G- P-
G+ P- 6	1	5
G- P+ 13	5	8

In table 3 at last the loss percentage of the two methods for toxicity testing is shown, calculated on the number of the toxic strains found

in the primary examination. It is hence clear, that the methods used for this examination showed about the same values — P 5.3 % and G 6.3 %.

Table 3.

Loss percentage of in vivo and in vitro methods calculated on the number of the toxic strains found in the primary investigation.

	Number of toxic strains	Percentage loss	
		P	G
Group 1	133	9,1	6,0
Group 2	202	3,0	6,5
Total	335	5,3	6,3

G. guinea-pig test

P. plate test

Finally, it may be added that in the cases where a quantitative estimation of the toxin-producing capacity of the diphtheria strains is not necessary, but only information of the toxicity or non-toxicity of the strain is wanted, the modification of the in vitro method, which has been described in model test 7, is better suited for routine work. Though up to date there do not exist results of larger examination series with this technique, it seems to constitute, if one may judge from present experiences, a reliable in vitro method. There exists both through interference and cross phenomena a possibility of making a rapid distinction between »false« and genuine toxicity reactions in the plates.

Summary.

After the description of some model experiments, there is given an account of the results of a simple in vitro method, compared with in vivo tests, for testing the toxin-producing capacity of diphtheria bacteria.

DISCUSSION

SVEN GARD: Ouchterlony's method offers some interesting possibilities for study of the mechanism of the flocculation reaction. A remarkable feature is the extraordinary sharply defined floccules that appear in Ouchterlony's medium — as compared to the relatively great flocculation zone in fluid media. Another problem that may be tackled with Ouchterlony's technique is the disputed question about the nature of the false flocculation.

THE LYMPHADENITIS PICTURE IN MAN WITH VARIOUS TYPES OF BACTERIAL INVASION

By N. Ringertz and C.-A. Adamson.

The study on which we are going to report has been based on a series of necropsy cases in which the lymphoglandular system was systematically examined both bacteriologically and histologically.

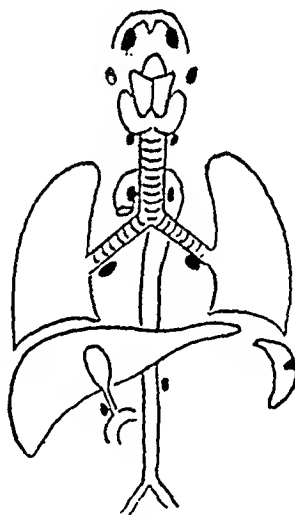


Fig. 1.

Briefly, the procedure consisted in excision, without special aseptic precautions, of whole lymph glands from the sites indicated in Fig. 1. In addition to the lymph nodes the tonsils were removed as well as a portion of the spleen and, in special cases, portions of other organs considered of interest. Furthermore, in all the cases blood plates were inoculated with material taken from the trachea and the main bronchi under, as far as possible, sterile conditions. The excised lymph glands, tonsils, and organ portions were three times singed with alcohol as soon as this could be done after completing the ne-

crepsy, and subsequently incised with sterile instruments. From the interiors of lymph glands or organ portions, aerobic and anaerobic cultures were made on blood plates and in broth. In the majority of instances also Löwenstein's medium was used. Material having been obtained for culturing, the lymph glands and organ portions were fixed with formalin and prepared for histological examination. In a fair number of instances, the results of cultures made during the life of the subject, serological titres and other clinical findings afforded valuable supplementary information.

As regards the composition of the series, it can be stated that the cases are rather evenly distributed over the following groups: (i) tuberculosis; (ii) various non-tubercular medical conditions; (iii) sudden death of subjects previously healthy (control group comprising street accidents, suicides, etc.). We do not intend to give a detailed presentation of the evidence afforded by the series as to the infective picture within the lymphoglandular system associated with various diseases. Adamson has made preliminary reports on this subject, viz. on the one hand, before the Swedish Medical Association in February, 1946 and, on the other, at the Meeting of Phthisiologists held at Gothenburg in June, 1946. However, it should be pointed out here that the source of error possibly entailed by the action of putrefactive micro-organisms apparently plays an amazingly slight rôle, and further that the group, *Control Cases*, as a rule presented negative culture findings as well as absence of more well-marked histological indications of lymphadenitis. It must also be considered that particularly in the group, *Tuberculosis*, there is a high incidence rate of major infection of the lymphoglandular system, i. e. principally by staphylococci and streptococci.

What we propose to report in the present paper is a study of certain variations in the non-specific inflammatory picture displayed by the lymph glands. There is apparently a correlation between these variations and various common types of bacteria, such as streptococci, staphylococci, *Esch. coli*, and pneumococci. An essential feature of the non-specific lymphadenitis picture is the so-called sinus catarrh. This is manifested by considerable proliferation and swelling of the sinus endothelium, the sinuses in cases of severe irritation appearing distended, engorged with large cells rich in cytoplasm and round or polygonal in shape, the nuclei of which are rather small and round. These cells show more or less conspicuous signs of phagocytic activity, i. e. ingested erythrocytes, leucocytes, and pigment, e. g. haemosiderin and carbon pigment. Polynuclear leucocytes usually play a minor part in the picture of sinus catarrh, but are always present in small numbers. In certain instances the latter cells are abundant, however. Another phenomenon which, though known, is but sparingly discussed in the literature, is the so-called plasmocytic response. In the pulpa of practically every irritated lymph gland occasional cells occur which are larger than lymphocytes, containing a fairly large

nucleus rich in chromatin and a rather sparse cytoplasm staining red with Unna-Pappenheim's stain. Transitions are noted between these cells and, on the one hand, anatomically typical plasma cells, as well as, on the other, the cells of the pulpa reticulum. The supposition readily suggests itself that these findings are due to differentiation of plasma cells out of reticulum cells; further, that the cells resembling reticulum cells and responding to Unna-Pappenheim's stain, are transitional forms. In immunization experiments on rabbits *Fagraeus* has succeeded in producing this cellular reaction *inter alia* in lymph glands, and has reported certain facts proving that the reaction is connected with antibody formation. In the following, the phenomenon will be designated as plasmocytic response without prejudice as to its biological significance. This response may be excessive in certain cases of lymphadenitis. The cells under discussion will sometimes be so abundant within the pulpa that they crowd out the lymphocytes from some areas, and in those instances they will also be found to intermingle in the sinuses with the large light sinus cells, from which they are easily distinguished.

In surveying our material — which seems to be unique with regard to the fact that in a large series, not only was there evidence available pertaining to the clinical conditions, serological titres, etc., but also that it was known which types of bacteria could be grown from every lymph gland examined — we soon found that there appeared to exist a remarkable relation between the lymphadenitis and infection pictures, viz. in the following respects: —

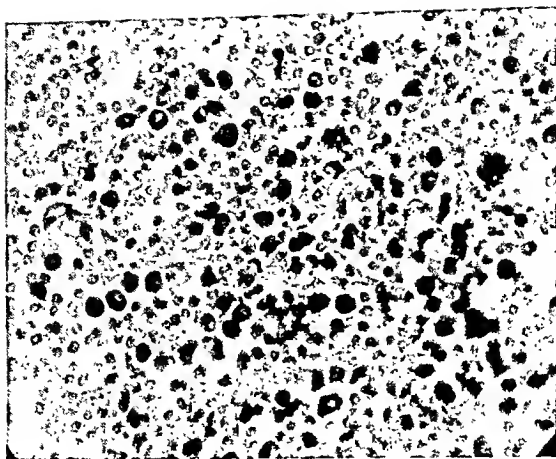
(1) Streptococcal infections (both by haemolytic and non-haemolytic streptococci) were associated with a particularly strong plasmocytic response, whereas the ordinary sinus irritation was rather inconspicuous in pure cases of streptococcal infection.

(2) In those cases where streptococcal infection was either absent or of minor importance, and where instead other organisms, viz. principally staphylococci and *Esch. coli*, predominated, there was no strongly marked plasmocytic response, the ordinary picture of sinus catarrh being instead evidenced proportional to the severity of infection. First and foremost, we found that ample growth of staphylococci without exception corresponded to severe sinus catarrh, whilst *Esch. coli* in certain instances seemed to be capable of producing powerful irritation of this type, but failed to do so in other cases. Since the glands in many cases were infected both by staphylococci and streptococci, a mixed picture of sinus catarrh and plasmocytic response was fairly common.

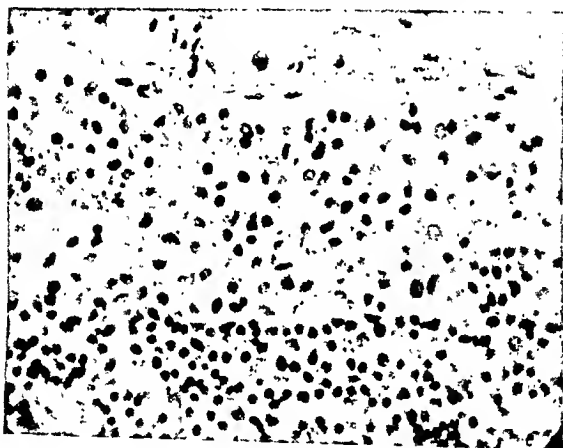
(3) A relation was thought to exist between pneumococcal infection and marked infiltration of the sinuses by polymuclear leucocytes.

Examples of the histological phenomena described above are given in Figs. 2—4.

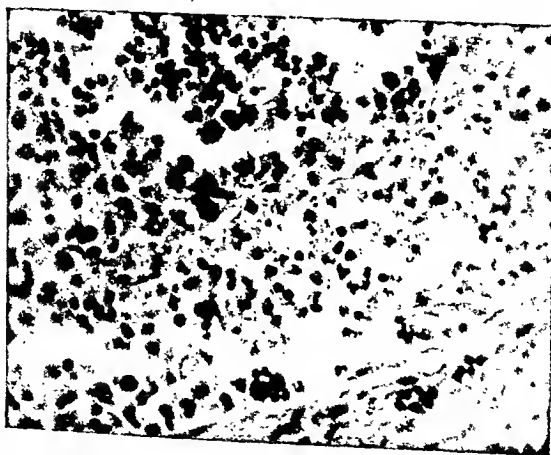
In order to investigate whether these observations made by us

*Fig. 2.*

Plasmocytic Response in a Pneumonia Case with Streptococci and Pneumococci (Unna-Pappenheim's stain).

*Fig. 3.*

Sinus Catarrh in a Case with Pure Staphylococcal Infection.

*Fig. 4.*

Sinus Showing Leucocytic Response in the Same Case as in Fig. 2.

Fig. 7.

Plasmocytic Response.

1.

Str.

31	7
14	42

2.

Staph.

30	22
15	27

3.

Coli

28	16
17	33

Sinus-Catarrh.

Staph.

31	22
5	37

4.

Coli

22	23
14	36

5.

Staph.

and/or

Coli

19	6
17	53

6.

Str.

19	19
16	40

7.

plained by the circumstance that, in certain instances, *Esch.coli* may grow on culture media without having been pathogenic, as is indeed to be expected. Table 7 relates to streptococcal infection versus sinus catarrh. A considerable scatter is noted, but with a certain concentration at lower right, due to the frequent occurrence of simultaneous streptococcal and staphylococcal infections.

Fig. 8.

Pneumococci	Leucocytic Response			
	0±	+	++	+++
Not demonstrated	58	11	3	3
Tonsil	2	1	1	1
Trachea, Bronchus and/or Lymph Gland ..	1	1	1	11

In Fig. 8 the correlation table is given for pneumococcal infection and ample occurrence of polynuclear leucocytes in the sinuses. In the histological gradation the same principle was used as above, viz. ++ and +++ indicating conspicuous responses. In grading the infections another principle was adopted, as for various reasons pneumococci are notoriously much more difficult to grow. The mode of gradation used will be seen from Fig. 8. Essential features of this table are, on the one hand, the marked concentration in the upper left cells 1 and 2, as well as, on the other hand, the fact that out of 14 cases with relevant pneumococcal infection, 12 show considerable leucocytic responses. Among the 6 cases at upper right, the leucocytic response is obscure in 2 cases; 4 were pneumonia cases, however, and in these *Haemophilus influenzae* was demonstrated, the presence of which possibly indicates a symbiosis with a pneumococcal strain refusing to grow on culture media. We have considered the possibility of the leucocytosis observed in the lymph glands simply being due to leucocytes being conveyed

to the glands from a pneumonic area rich in these cells. However, it was found that a considerable proportion of subjects with pneumonia and other suppurating pulmonary conditions were among the 58 cases at upper left, and that the 11 cases at lower right comprised 5 without pneumonia.

As was stated above, a large proportion of the series consisted of tuberculosis cases with lymph glands yielding in a high incidence rate both histological evidence of tuberculosis and growth on Löwenstein's medium. In practically all of these cases, there was at the same time an ample invasion of staphylococci and streptococci, frequently both. In those portions of the lymph glands which had escaped specific tuberculous inflammation, we observed a non-specific lymphadenitis picture with variation according to the foregoing. On account of mixed infection being present practically without exception, it is not possible to apply statistical methods to the question of whether tuberculosis is in itself of any importance for the type of the non-specific lymphadenitis picture.

In our opinion the results of this combined bacteriological and histological investigation carried out with a material comprising human lymph glands are apt to arouse interest. We feel that the numerical evaluation, having regard to the sources of error which can be supposed to exist, shows a reasonable degree of positive correlation between streptococcal infection and the phenomenon of the plasmocytic response; between infection by other organisms, principally staphylococci and partly also *E. coli*, and the reaction of the sinus catarrh type; and, lastly, perhaps also between pneumococcal infection and the accumulation of polynuclear leucocytes in the sinuses. At the present moment we are not prepared to discuss the biological significance of our observations. However, the result shows that it is well worth while to undertake a combined bacteriological and pathological investigation of this type with a series of necropsy cases.

DISCUSSION

ASTRID FAGRÆUS: Attempting to discuss the biological significance of the difference in cellular reactions, found by Ringertz and Adamson, I would like first to draw attention to the plasmacellular reaction. I have found the same type of reaction after injections of bacterial as well as non-bacterial antigens. After intravenous injection of the antigen reticulum cells in the spleen are differentiated to plasma-cells simultaneously with the appearance and increase in concentration of antibodies in the serum. The reaction in the spleen is confined to the red pulp, whereas the lymph follicles show very few plasma cells. In vitro studies show that only the red pulp is capable of forming antibodies in tissue cultures and this capacity depends on the stage of development of the plasma-cells when cultured. The conclusion is thus

drawn that antibody formation under these conditions is linked to the differentiation of plasma cells and that the plasma cell is the morphological manifestation of an intensively antibody-forming reticulum cell.

As I have found the same picture, also in the lymph nodes, after injections of different kinds of antigen, I can hardly believe that the plasmacellular reaction should be specific for the formation of antibodies against streptococci. The endothelial reaction found in lymph nodes with staphylococci might be partly or entirely a phagocytic reaction. The staph. c. toxin is very cell-toxic and Ringertz mentioned cells and cell débris deposited within the endothelial cells. That being the case there should be a difference in reaction if pathogenic or saprophytic staph. c. were found in the lymph nodes.

The opsonic effect of pneumococci is very well known and might explain the accumulation of leucocytes found in lymph nodes with pneum. c.

L. HEERUP: On estimation of the histological findings in lymphadenitis it is important to keep in mind that two types of changes may be present: 1) changes produced by immunizing processes and 2) changes due to the difference in the tissue reaction produced by the various bacteria.

As to changes of the first type, in Denmark, among others, Johannes Ipsen, Bjerneboe, Jens Bing and Gormsen have demonstrated that the number of plasma cells is greatly increased in immunizing processes, and they simply are looked upon as antibody producers.

As to changes of the other type, Menkin has demonstrated the characteristic difference in the tissue reaction to streptococcus and staphylococcus infections. In staphylococcus infections fibrin thrombosis takes place in the surrounding blood vessels and lymphatic, delimiting thus the extent of the inflammation, whereas in streptococcus infections fibrinolysis takes place, promoting the clinically well-known diffuse spreading of the inflammation.

Aseptic precautions are advisable in the extirpation of the lymph nodes, as the findings of concomitant bacteria, especially *B. coli*, undoubtedly are due to superficial contamination of the lymph nodes, the surface of which has not been sterilized thoroughly by the disinfection methods here employed.

T. PACKALÉN: As to the misgivings put forth in the discussion — that the bacteriological findings might be misleading either because the lymph glands have not been handled under observation of sterile precautions at the autopsy, or because of postmortal invasion of irrelevant bacteria from the outside — I should like to add a few remarks apart from what has been said already in favour of the reliability of the inoculating technique employed by Adamson:

When these investigations were started we had some doubt our-

selves as to the practicability of making reliable observations with the working methods planned. But our experiences have done away with all such doubt.

Adamson has already mentioned some of the reasons for this.

The bacterial findings which most readily might be suspected of originating from contamination or postmortal invasion consisted in strains of colon bacilli, staphylococci, α and γ streptococci. As to the colon bacilli, it is to be emphasized that — according to the findings reported by Henriksen in Norway and by our investigators here in Sweden — they occur far more frequently than has been assumed before, in the upper respiratory passages too. Practically all the staphylococci isolated from the lymph glands were of the aureus type, hemolysin- and coagulase-positive, *i. e.*, pathogenic. Like other strictly saprophytic microorganisms inhabiting the upper respiratory passages (diphtheroid rods, saprophyta neisserior, etc.) staphylococcus albus was not found in the lymph glands. It is difficult to imagine a selective postmortal invasion of the lymph glands by potentially and manifestly pathogenic bacteria alone, with suppression of the saprophytes. It is even more difficult to understand any selective contamination in this direction owing to the non-aseptic autopsy. On the other hand it seems quite natural that in a living organism the defensive forces would be able more easily and effectively to eliminate the evading saprophytes than the pathogenic bacteria.

N. RINGERTZ: In our opinion the technique here employed is adequate with regard to sterility. In the group of »controls« the examined glands were found to be sterile in many of the cases. Control experiments have been carried out in order to ascertain whether flaming of the glands will kill the bacteria inside. A risk of this was found to be present in very small glands (smaller than hemp-seed), but glands of this size have been exceptional in our material. Our studies furnish no evidence to the effect that the glands have been invaded post mortem by bacteria from other parts of the body to any great extent — at least not as far as the lymph glands of the respiratory passages are concerned. Growth of *B. coli* from these glands will undoubtedly be due to premortal invasion. It reminds of the findings reported by Henriksen and Paekalén, showing colon bacilli to be present often in the sputum and throat swabs from patients.

It is possible, however, and even likely that bacteria present in the glands at the death of the patient will multiply post mortem. This is indicated to some extent by a series of examinations — not mentioned in our paper — of mediastinal glands removed at thorax operations. Generally the cultures from these glands showed less abundant bacterial growth than did the cultures from autopsy material even though the histological picture of the former indicated considerable inflammatory processes.

STUDIES ON THE AGGLUTININ FORMATION IN BRUCELLAR INFECTION OF THE GENITALS OF THE BULL

By N. O. Christensen.

In their comprehensive studies on brucellar infection in the bull, with a special view to the bacteriological and immunological aspects of the semen, *Bendixen & Blom* found that in brucella-infected bulls with localization to the genitals the presence of agglutinins for *Brucella abortus* may be demonstrated in the seminal plasma, and that the agglutinin titer of the seminal plasma often is considerably higher than the corresponding titer of the blood plasma. This fact together with the circumstance that brucella-infected bulls without focal processes in the genitals very well may show a blood titer of a certain height while no agglutinins can be demonstrated in the seminal plasma, indicate that the formation of these antibodies takes place locally in the inflammatory processes produced by the *Brucella* bacteria.

This paper is a preliminary report of studies aimed especially to find a morphological basis for this antibody formation by means of histological examination of such brucella-infected organs.

My material comprises the genitals of 16 bulls (see Table 1), 13 of which were found at the above-mentioned examination to be infected with *Brucella abortus*, while 2 bulls (Nos. 115 and 116) were infected experimentally by injection of virulent *Brucella* bacteria into the vesicular glands, and 1 bull (No. 57) originated from a material of slaughter-house bulls.

After the bulls were killed, the genitals were brought to the laboratory as soon as possible, and here an agglutination test for *Brucella abortus* was performed on secretions pressed out — and if necessary, centrifuged — from the ampullae ductus deferentis and the glandulae vesiculares, besides, in some cases, on exudate from the sequestral cavity in the presence of necrotizing orchitis. In addition, the organs were examined thoroughly, macroscopically and histologically.

Table 4.

Bull No.	Date	Blood titer	Sperm-plasma titer	Secretion right ampulla ductus deferentis	Secretion left ampulla ductus deferentis	Secretion right glandula vesicularis	Secretion left glandula vesicularis	Abscess content from orchitis mortificans	Pat. anat. Diagnosis	Duration at least
57	1946 12/2	1:640	1:2560	—	—	(1:20)	1:10240	—	Vesiculitis sin.	7
109	6/6	1:20	1:20	—	—	0	1:20	0	Orchitis dex. Vesiculitis duplex	7
110	20/6	1:80	1:160	1:2560	0	1:2560	0	—	Vesiculitis el. Ampullitis dex.	2 mths. 5
111	20/6	1:80	1:160	—	—	0	1:5120	—	Vesiculitis sin.	4
113	17/7	1:10	1:40	1:640	0	0	0	—	Ampullitis dex.	4
114	10/10	1:40	1:640	—	—	0	1:1280	—	Vesiculitis sin.	6
115	10/10	1:320	1:320	1:100	0	1:640	0	—	Vesiculitis el. Ampullitis dex.	5 5
									Ampullitis sin. Vesiculitis duplex	5 5
116	10/10	1:160	1:640	0	1:400	1:3200	1:3200	—		
117	20/11	1:160	1:640	—	—	0	1:5120	—	Vesiculitis sin.	5
118	5/12	1:100	(1:40) 0	0	0	1:10240	0	—	Vesiculitis dex.	7
122	1947 9/1	1:10	0	—	—	0	0	—	Vesiculitis sin.	2 years
123	12/1	1:1280	1:10240	1:2560	1:5120	1:20480	1:5120	—	Vesiculitis el. Ampullitis duplex	5-6 mths.
124	5/2	1:320	1:640	(1:10)	1:2560	0	1:1280	—	Vesiculitis el. Ampullitis sin.	1
125	21/4	1:80	1:320	0	0	0	1:10240	—	Vesiculitis sin.	
126	8/5	1:2560	1:640	(1:40)	1:1280	1:5120	(1:40)	1:10240	Orchitis sin. Vesiculitis dex. Ampullitis sin.	1
128	6/6	1:160	1:20	0	1:160	0	0	1:320	Orchitis sin. el. Ampullitis sin.	6

Survey of the Agglutinin Titers for Brucella abortus shown by the Examined Bulls in Blood, Seminal Plasma and Various Secretions and Exudates. Further Pathologic-anatomical Diagnosis, together with the Approximate Duration of the Illness.

The lowest dilution employed in the agglutination tests has been 1:10; 0 means absence of any agglutination. — means that no agglutination test was made. In the case of Bull 118 the value recorded in parenthesis in the column of seminal plasma refers to an earlier examination of the semen. For further details, see the text.

Table 1 gives the outcome of these examinations as well as the dates on which the respective bulls were slaughtered and the results of the last blood and seminal plasma examination prior to the slaughtering of the animals. In the individual cases, furthermore, the approximate duration of the illness is given insofar as data on this point have been available. Thus the duration recorded has to be looked upon as a minimum figure. Generally it may be established that here we are dealing with more or less chronic cases of the infection, with a duration of illness varying from about 3 months to about 3 years.

From Table 1 it will be noticed that the titer of the blood plasma and the titer of the seminal plasma have lain at somewhat different levels, depending on the localization and duration of the infection. As a rule, however, the titer of the seminal plasma has been higher than that of the blood plasma when the ampullae ductus deferentis and/or glandulae vesiculares have been attacked. In those cases where also orchitis has been present (Nos. 126 and 128) the titer of the blood plasma was found to be rather considerably higher than the titer of the seminal plasma. Apart from a few cases that will be mentioned below, a very good agreement has been found between the occurrence of pathological changes in the organs brought about by *Brucella abortus* and the finding of agglutinins of a certain titer in the corresponding secretions or exudates, and usually this titer has been considerably higher than the corresponding blood and seminal plasma titers.

In 3 cases (Nos. 57, 124 and 126) some low titer values are recorded in parentheses. No pathological changes were found in the organs here concerned, and presumably the occurrence of agglutinins in these cases has been due to a slight admixture of secretion or blood with a high agglutinin content, even though great care has been taken in obtaining the secretions from the various organs in as pure a form as possible. On the other hand it must not be neglected that the reason may be the presence of small pathological processes due to brucellar infection which have not been detectable macroscopically and have not been encountered with in the histological sections made.

In two bulls (Nos. 109 and 122), in spite of typical pathological changes, no agglutinins were found, or the agglutinin content was very low. In bull No. 122 the infection had lasted for about 3 years, and in this animal it had been possible to ascertain a continuous fall in the agglutinin content of the seminal plasma as well as of the blood serum, so that in this case the age of the pathological processes may explain the lack of agglutinins. Bull No. 109, on the other hand, showed entirely deviating features with regard to the agglutinin contents of the blood and seminal plasma as well as to their local occurrence. In contrast hereto, the histological picture of the organs attacked was typical; and, furthermore, *Brucella* bacteria were isolated from these organs. So, on the basis of the hitherto available ex-

periences, it will not be possible to offer any adequate explanation of this phenomenon.

In the ampulla ductus deferentis the bruceellar inflammation brings about a more or less pronounced enlargement of the organ, and the tunica muscularis becomes greyish-white in colour because of the connective tissue infiltration.

Bruceellar inflammation of the glandula vesicularis — vesiculitis — is also associated with a considerable enlargement and hardening of the organ, with effacement of the normal lobulation. Further, the capsula is thickened with connective tissue infiltration, and often this new-formation of connective tissue involves also the plica urogenitalis. On section, the cut-surface indicates that in principle the pathological changes here observed may have developed in two ways. In the gland as a whole we meet with an extensive and marked increase in connective tissue as well as cellular infiltration of the capsule and interlobular strands of connective tissue. But, in addition, several specimens show relatively large and irregular areas of necrosis in the central parts of the gland. At the time of the examination the sequestrum has become detached and the sequestral cavity, containing yellowish lumpy pus, has been communicating with the excretory system of the gland. The membrane of the abscess has consisted in a thin layer of preserved glandular tissue that has been the site of the above-mentioned indurative changes.

The 3 cases of orchitis here examined have been typical examples of diffuse gangrenous orchitis, in which the testis as a whole has undergone necrosis with demarkation, so that the more or less dissolved sequestrum has been embedded in a serous exudate with lumps of pus or in a more homogeneous purulent exudate. The sequestral cavity has been surrounded by a layer of fibrous granulation tissue, measuring a centimeter or more in thickness and comprising also the thickened tunicae, which have been matted together. On the side involved the tunica dartos has been considerably hypertrophic in comparison to the normal structure.

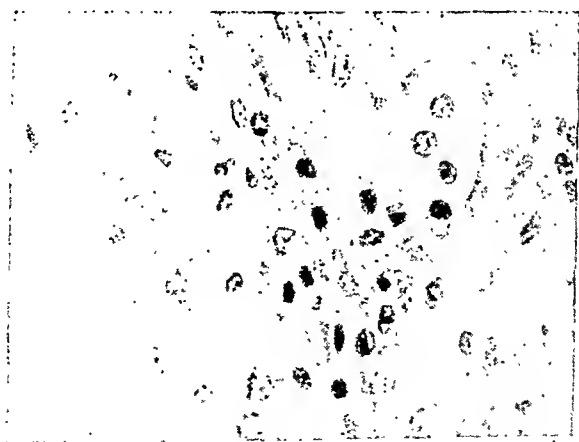
The *histological picture* of these bruceellar inflammatory processes — all of which, as mentioned, have been of a more or less pronounced chronic character — with variations according to the organs involved, are on the whole consistently characterized by new-formation of connective tissue and by cell infiltration, which together contribute greatly to the effacement of the normal histological features.

In the specimens of diffuse *vesiculitis* a very marked increase in the connective tissue of the capsule is noticed as well as an increase in the interlobular and intralobular streaks of connective tissue, so that the collagenic fibrils more or less have masked the smooth musculature.

The cell infiltrations consist in part in fairly well defined heaps of cells in the thickened streaks of connective tissue. It is a striking

*Fig. 1.*

Bull 118. Glandula vesicularis. Border of a glandular alveolus with contents rich in cells and proliferation of the glandular epithelium, which, besides, is forced out into the lumen by the underlying cell infiltration.
 van Gieson-Hansen stain. Magnif. $\times 200$. 2:3.

*Fig. 2.*

Bull 118. Glandula vesicularis. Interalveolar connective tissue septum with typical plasma cells. To the left, well preserved glandular epithelium with wandering cells.
 van Gieson-Hansen stain. Magnif. $\times 900$. 2:3.



Fig. 3.

Bull 57. Glandula vesicularis. Border of sequestral cavity in necrotizing vesiculitis. Note the strongly proliferating glandular epithelium that delimits the sequestral cavity above; beneath this, pronounced cell infiltration. van Gieson-Hansen stain. Magnif. $\times 125$. 2:3.

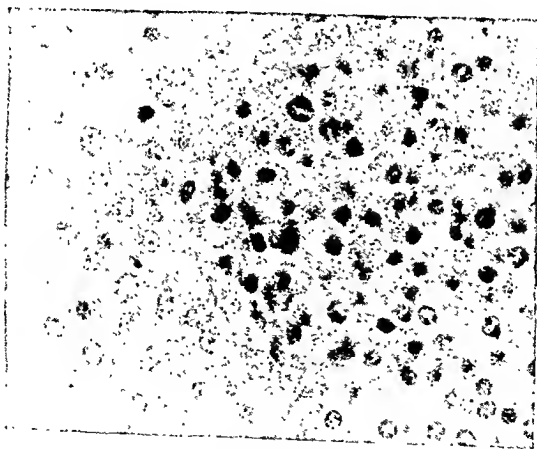


Fig. 4.

Bull 57. Glandula vesicularis. The same specimen as Fig. 3, at higher magnification. To the left, the proliferating epithelium; beneath this, typical plasma cells. van Gieson-Hansen stain. Magnif. $\times 900$. 2:3.

feature, however, that most of these infiltrations are found in close connection with the glandular alveoli, appearing as thick cellular borders just beneath the glandular epithelium, which in many cases is forced into the lumen, resulting in the obliteration of the latter (Fig. 1).

The cell infiltrations are made up chiefly of plasma cells at various stages of development characterized by a cytoplasm, which is stained red by pyronine, and as to the fully developed plasmacells, by excentric situation of the nucleus and a more or less distinct marginal position of the chromatin substance — cart-wheel nucleus —, besides juxta-nuclear vacuole (Fig. 2). In addition, we meet with lymphocytes, histiocytic cells and varying amounts of granulocytes, heterophil as well as acidophil.

The epithelium of the glandular alveoli may consist in one layer of cells as normally, but in many cases it has undergone considerable proliferation, being then made up of several layers of polygonal cells. Through the epithelium there is a very marked migration of cells with a round dark nucleus. In a few cases even typical plasma cells are seen between the epithelial cells or in the glandular lumen.

Often the glandular alveoli are considerably distended by an exudate very rich in cells. But in some cases the lumen of the alveoli appears very small or, occasionally, quite obliterated — owing to proliferation of the glandular epithelium and to the fact that the epithelial cells are forced into the lumen by the cell infiltration.

In those cases where the glandula vesicularis has undergone central necrosis we meet with the characteristic feature that the glandular epithelium of the preserved alveoli grows very vigorously, protruding and bordering the sequestral cavity as a thick cellular membrane consisting of several layers of polygonal cells. Beneath this epithelium there is a very pronounced cell infiltration, made up chiefly of plasma cells at different stages of development, lying as a pavement, with fine collagenic fibrils between the individual cells. Here, too, there is a pronounced emigration of cells through the epithelium (Figs. 3 and 4).

In the *ampulla ductus deferentis* the pathological changes may be seen partly in the mucous membrane of the ductus deferens itself, partly in the glands in the wall of the duct. A typical instance of isolated brucellar ampullitis has been described previously by Bendixen. The changes observed in the glands correspond fairly well to the changes described in the cases of diffuse vesiculitis, although there appears here to be a greater tendency to the occurrence of granulocytes. Besides, at the same time there is a considerable new-formation of connective tissue and cell infiltration in the stratum proprium corresponding to the mucosa of the ductus deferentis. In some cases the mucosa may protrude polypously into the lumen of the duct, so that this loses completely its typical stellate form. In the center of these mucosal protrusions there is a pronounced cell infiltration, consisting chiefly of plasma cells with some admixture of granulocytes.

In several cases the last-mentioned changes in the mucosa ductus deferentis have been the only abnormal features seen in the ampullae.

In *gangrenous orchitis*, with the sequestrum embedded in pus, the sequestral membrane facing the cavity is apparently a syneutic membrane of large cells with vacuolized acidophil cytoplasm and large vesicular nuclei. Beneath this membrane, cell infiltrations are seen that correspond fairly closely to those described in cases of vesiculitis, and which consist chiefly in plasma cells. Besides, in this zone, there are also remnants of a few seminiferous tubules with completely deteriorated, partly calcified, contents with some foreign-body giant cells. The external part of the sequestral membrane is made up of fibrous connective tissue, in which small scattered cell infiltrations are seen.

The observations here presented will further corroborate the assumption that in these forms of brucellar infection the agglutinin formation takes place *locally* in the organs involved. The most characteristic feature common to the pathological changes here observed is *the conspicuous occurrence of plasma cells and a close connection between these cells and the glandular epithelium and demarcation membranes* — a fact which highly lends support to the hypothesis that these cells play an important rôle in the antibody formation, perhaps indeed are the real antibody producers.

Summary.

A brief preliminary report is given of the findings obtained in 16 bulls with brucellar infection of the genitals.

Agglutination tests for *Brucella abortus* are made on secretions or exudates pressed from the ampullae ductus deferentis and the glandulae vesiculares and, in some cases, taken from the sequestral cavity in gangrenous orchitis.

The results thus obtained are compared partly with the corresponding agglutinin titers of the blood and seminal plasma, and partly with the pathologic-anatomical findings in the organs mentioned. With a few exceptions, described in detail, these comparisons have shown a quite convincing relation between the occurrence of agglutinins and the finding of pathologic-anatomical changes in the corresponding organs, the most characteristic common feature of which consists in pronounced cell infiltrations made up chiefly of *plasma cells* in close connection with the glandular epithelium and demarcation membranes.

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NO DISCUSSION

TOXOPLASMOSIS CANIS*)

By I. P. Sjötte.

A parasite, the life cycle of which is still unknown, is the parasite first described in 1908 by Nicolle and Manceaux in a north African rodent — *Ctenodactylus gondii* — which they found had a lengthy crescentic body, one end of which was much more pointed than the other, and the length of which was given as 5—7 μ and the width as 3—5 μ . They found the nucleus to be round or oval and located centrally in the parasite.

In Brazil, *Splendore*, at the same time, found quite similar parasites in rabbits. His indication of the size is slightly different, but still, in all essentials it is the same; in addition, however, *Splendore* found the parasites to be grouped in cyst-like formations which later on came to be known in the literature as *Pseudocysts*.

Morphologically, similar parasites have been found by other observers in a number of different animals — both rodents, beasts of prey, ruminants and monkeys — thus, in the literature there are instances on record of its presence in guinea-pigs and mice, further, in a cat and in number of cases in dogs; further, one case in a sheep and one in a chimpanzee. According to the reports in the literature it would seem that the incidence of Toxoplasmosis is greater in birds, in which the first case was found in sparrows in 1900 (described by *Leveran*). Later Toxoplasmosis has been observed in birds of different kinds (pigeons, chicks, canaries, siskins, and many other varieties of birds).

Also in man a number of cases of Toxoplasmosis has been reported. The first case observed dates back to 1913 and was found by *Castellani* in a 14 years-old Singhalese. 10 years later a case occurred in an 11 month old boy in Prague, later, in 1927, a case was observed in Rio de Janeiro, and in the end of 1930 several cases were demonstrated in adults as well as in children in North America and South America.

A great number of the cases in man have simulated meningo-encephalites, but besides, inflammatory changes have been found in other tissues, such as the myocardium and the skeletal muscles, and also in interstitial and fibrino-purulent pneumonias. In liver and spleen

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small necroses have been observed and in various locations in the altered tissues the presence of toxoplasmas has been demonstrated. More significant changes in the gastro-intestinal canal seemingly have not been observed in those human beings whose cases have been reported. Only in one case, published by *Pinkerton and Weinmann* from Lima, petechiae were found in the gastric mucosa, and mesenteric retroperitoneal and inguinal lymph nodes were enlarged to 2 or 3 times their normal size, and the site of haemorrhages and necroses.



Fig. 4.

Stomach cut open, showing the well developed folds of the mucous coat and numerous small ulcerations, partly between the folds of the mucosa, partly on the ridge of them, the ulcerations being most numerous in the centre.

In the cases in animals reported in the literature the conditions has, from a pathological-anatomical point of view manifested itself — briefly outlined — as a non-suppurative encephalomyelitis with toxoplasmas in the inflamed areas of the brain, as it has been observed in a sheep, or as a necrotizing and ulcerous enteritis and necrotizing lymphadenitis, splenitis and hepatitis, as it has been observed in dogs and, in one case, in a cat. Further, in this cat, multiple pneumonic foci were found to be present with considerable growth of the alveolar epithelium with adenomatous neoplasm-like formations, with demonstrable toxoplasmas in epithelial cells and monocytes.

Geographically, it was originally in the warmer climates that this condition was encountered, but more recent reports about the condition have, however, shown that the condition may also occur in the



Fig. 2.

Aggregation of toxoplasmas in endothelial cells (opposite arrow) extending upwards to the right in the picture.



Fig. 3.

An agglomeration of toxoplasmas in epithelial cell of a stomach gland. South hereof — out of focus — a similar aggregation.

more temperate climates. This is seen from cases reported from Germany, France and England, and in quite recent years, also from the state of New York. From as northerly places as Scandinavia no verified cases have to my knowledge occurred until now, neither in birds, nor in mammals. In the literature, at any rate, no such cases are on record.

The first case of verified toxoplasmosis here in Denmark was observed in a dog suffering from its illness in the middle of a lengthy and uncommonly severe period of frost. The illness had only lasted for 8 days and resembled to a no slight degree canine distemper with pneumonia, but was without nervous symptoms.

The autopsy examination showed a great number of ulcerations in the stomach and small intestine, with numerous toxoplasmas in the tissue of the structures immediately surrounding the ulcerations. In the lungs numerous miliary to supermiliary nodes, evenly distributed, were seen, which were found to be a miliary eroupous pneumonia with a small number of toxoplasmas in the macrophages in the alveoli.

The lymph nodes of the affected organs were very considerably enlarged and on the cut surface rather presented the appearance of lymph nodes with intensive tuberculous caseation. In the fibrinous infiltration and markedly necrotic areas of the tissue there were moreover a few living cells with a great number of toxoplasmas.

No inoculation and examinations were made of the virulence of the parasites, as the case — so to speak — rather took one by surprise, and fresh material therefore was not reserved for this purpose, but the intensive inflammatory reaction around the toxoplasmas and the characteristic appearance of these, which is best seen by staining with Harris hæmatoxylin, and otherwise the absence of other micro-organisms and also eosinophilia of the tissue, show that the lesion must be a *Toxoplasma*.

To judge from this case and also from the cases reported in the literature the infection must be taken, in any case as far as the animals of prey are concerned — to have occurred via the gastrointestinal canal. Whether there are non-generalized cases of toxoplasmas, is for the time being an open question. It has not been possible to obtain any information as to from where the animal here dealt with received its infection, but the case shows that the infection is there, and that we must reckon with its presence in Scandinavia.

Experimental infection and biological researches on toxoplasmas both from man and from animals and birds — these researches have been carried out in America — seem to indicate that the toxoplasmas from animals and birds and possibly also those from man are identical.

I therefore consider that the case is of a considerable interest to comparative pathology.

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DISCUSSION

See under the following paper.

HUMAN TOXOPLASMOSIS AN ACCOUNT OF TWELVE CASES IN SWEDEN

By J. Henning Magnusson and F. Wahlgren.

It has long been known that *Toxoplasma*, a genus of protozoa, is pathogenic for certain lower animals (Splendore, Nicolle and Mancaux, Levaditi and co-workers, Mooser). During recent years Sabin and Olitsky in particular have considerably increased our knowledge concerning this micro-organism. They succeeded in isolating a strong pathogenic strain of *Toxoplasma* from a guinea-pig. This strain proved to be pathogenic for mice, rabbits, guinea-pigs, rhesus monkeys, hens and chickens. The infection could be transmitted by oral, intranasal, intracutaneous, subcutaneous, intracerebral and intravenous routes. Contact infection was studied in mice. It occurred only when starved animals were allowed to feed on others that had recently died of the experimental disease. *Toxoplasma* was shown to be an obligate intracellular parasite, only multiplying in living cells. Facts hitherto ascertained appear to indicate that it divides by binary fission and that multiplication can occur not only in monocytes and endothelial cells, but also in practically all kinds of tissue cells. Sabin and Olitsky also found that toxoplasmic infection in rhesus monkeys gave rise to neutralizing antibodies, whereas rabbits as a rule became immune without the appearance of such specific antibodies. Olafson and Monlux have recently implicated *Toxoplasma* as the cause of a fatal disease in dogs, and have described cases in cats and sheep, thus implying a possible origin of infection in man. Perrin, Brigham and Piekens have demonstrated toxoplasmic infestation in 8.7% of a group of wild rats, studied in the south-eastern states of the U. S. A.

The morphology of the *Toxoplasma* parasite is well elucidated. In the fresh state and in air-dried films, the length is up to 4—7 μ , the breadth up to 2—4 μ . In slides from fixed tissue it can be considerably smaller. Immediately after division, the form is crescentic, in later stages of development it is piriform, oval or round. The parasites are highly-organized, with distinct cytoplasm and nuclear

chromatin without a nuclear membrane. The nuclear chromatin constitutes approximately one-third of the parasite and gives a strong positive Eoulsen reaction (Sabin). Toxoplasma is, like other protozoa, gram-negative. In the tissue it can be found singly, in clumps, within cells, and as so-called cysts or pseudo-cysts, which represent host cells entirely filled by micro-organisms.

In 1939 it was finally proved that this parasite can also cause human infection and give rise to the disease called toxoplasmosis. In that year Wolf, Cowen and Paige succeeded in isolating from a child a strain of Toxoplasma which was highly virulent to the ordinary laboratory animals. The patient died at the age of 31 days from »congenital« encephalo-myelitis. Comparative experiments made by these authors and by Sabin showed that this human strain of Toxoplasma differed neither biologically nor immunologically from the strain isolated earlier by Sabin from a guinea-pig.

Human toxoplasmosis can be either congenital or acquired. The age factor appears to influence the clinical picture considerably, and very varying clinical types have been described.

The most prominent characteristic in the congenital form of the disease is the simultaneous appearance of symptoms from the eyes and the nervous system at or shortly after birth. The ocular lesions are bilateral and consist of multiple foci of chorioretinitis, which appear consistently to be localized to the macula region. Less regularly occurring symptoms are microphthalmus, nystagmus and ocular paresis. In the nervous system we find hydrocephalus, or microcephalus, convulsions or other psycho-motor disturbances. Finally, there is a characteristic change, demonstrable roentgenologically, namely multiple intracerebral calcified foci. Occasionally, prolonged icterus has been observed, as well as enlargement of the liver and spleen. The cerebrospinal fluid shows xanthochromia, with an increased number of cells and a raised protein content.

Foetal hydrocephalus has been demonstrated as the result of a toxoplasmic encephalo-myelitis in a stillborn foetus.

Congenital or neonatal toxoplasmic encephalo-myelitis often has a fatal outcome during the first few weeks or days of life. The lesions found in the central nervous system in such cases consist principally of localized destruction of the cerebral tissue, most commonly in the vicinity of the ventricle system, accompanied by considerable accumulation of inflammatory cells and calcium, leading to hydrocephalus. In addition, a focal chorioretinitis is regularly found. At times lesions are also found in other organs, such as the myocardium, liver, etc.

Sometimes, however, the disease can progress without any definite symptoms to the end of infancy or even up to the earlier years of childhood, when symptoms of residual damage appear. In those patients who survive the earlier stages, hydrocephalus remains or increases. The chorioretinitis regresses, but causes permanent impair-

ment of vision and often searching nystagmus. Mental development shows a tendency to retardation. During recent years, a number of cases have been traced with residual symptoms of such a type that there has probably been an earlier toxoplasmic infection. In a great number of these cases it was possible to verify the diagnosis on further investigation (Vail, Strong and Stephenson, Heidelman, Johnson, etc.).

The excellent contributions made by Wolf and his co-workers in a series of publications form the basis of the major part of our present knowledge of the congenital form of toxoplasmosis. Studies on such cases have also been published by a number of other writers (Sabin, Levin and Moore, Steiner and Kaump, Zuelzer, Sailer, Pratt-Thomas and Cannon, Bamatter). With our present knowledge as a background, it has been possible moreover to prove that a number of cases published earlier have, in all probability, been cases of congenital toxoplasmosis.

Janků in Czechoslovakia, thus described in 1931 parasites in the retina of a child who had suffered from hydrocephalus and ocular lesions since birth. Levaditi (1928) was of the opinion that the parasites were *Toxoplasma*. Torres in Brazil demonstrated in 1927 intracellular parasites in the central nervous system, myocardium, skeletal muscles and subcutis of a child who had died on the second day of life and had exhibited general convulsions. In 1938, Wolf and Cowen identified the case as toxoplasmosis. Cornelia de Lange in Holland described in 1929 the case of a child with hydrocephalus and encephalo-myelitis, with a fatal outcome at the age of 4 months. Wolf and Cowen in 1940 undertook a restudy of the sections from this case and then found *Toxoplasma*-like parasites. Hartig in Boston described in 1934 a premature infant in which a bacterial infection had caused death at the age of 25 days. The central nervous system, lungs, myocardium and adrenals from this case contained parasites which the author classified as *Sarcocystis*. Pinkerton and Weinman were, however, able at an examination in 1940 to identify them as *Toxoplasma*. Riechter in Chicago published in 1936 the report of a case which had shown fever, convulsions and opisthotonos with a fatal outcome at the age of 7 weeks. The fact that the lesions in the central nervous system were chronic and calcified showed that the changes were of long duration. On after-examination of this case in 1938, Wolf and Cowen found *Toxoplasma*-like parasites.

Two further cases of earlier date can be mentioned here. One was published by Wohlwill in 1921 and the other by Fischl as early as 1897. It is true that neither of these cases can with certainty be identified as toxoplasmosis, but the clinical picture shows a striking similarity to that found in this disease.

The acquired form of human toxoplasmosis appears, judging by the small number of cases published hitherto, to show very varying clinical pictures. In 1941 Sabin, who has made great contributions to the knowledge of this disease, published the report of two such cases from Cincinnati. Both were boys aged 6 and 8 years respectively, who developed atypical encephalitis. The clinical picture was characterized by fever, generalized convulsions, asthenia of the extremities, disorientation, pleocytosis — although such signs of meningeal irrita-

tion as rigidity of the neck and Kernig's sign were lacking — and lack of symptoms of injuries to the cranial nerves. One child died 30 days after the onset of the disease. The other recovered, with no residual clinical symptoms. In 1947 Robinson described the case of a 9-year-old Italian girl, who became ill with hyperpyrexia, strabismus, and chorioretinitis. The abdominal, knee and Achilles reflexes were lacking. Kernig's and Babinski's signs were positive. Both free and encysted parasites, which the author classified as *Toxoplasma*, were found in the cerebrospinal fluid. Neither inoculations on laboratory animals nor serological examinations appear to have been made. The patient was treated with sulphathiazole and emetine and recovered. In 1943, Guimarães described a case from Brazil. This was an 18-year-old negro who became ill with fever, rigidity of the neck, dysphasia and paralysis of the lower extremities. He died 37 days later, and changes typical of toxoplasmosis were found in the cerebrum, the spinal chord, the pericardium and the kidneys. We can also, perhaps, include a case from earlier literature. In 1929, Coulon described parasite-like formations which — on account of their size — he called *Enzcephalitozoon brumpti*, in the cerebrospinal fluid of a 17-year-old Corsican, who died of meningitis after 3 days.

In 1940, Pinkerton and Weinman reported a case of toxoplasmosis in a 22-year-old Peruvian. In this case, which had a fatal issue, the clinical signs were obscured by a simultaneous infection with *Bartonella bacilliformis*. In the following year, Pinkerton and Henderson published an account of two fatal cases aged 43 and 50 years. By means of this work it was clearly established that toxoplasmic infection in adults can be manifested in a syndrome simulating the typhus-spotted fever group, and is characterized by fever, cutaneous exanthema and atypical pulmonary involvement. Syverton and Slavin demonstrated *Toxoplasma* in the muscle tissue in a biopsy from a 65-year-old patient during his convalescence from an acute febrile illness with diarrhoea and tenesmus, as well as dull pains in one elbow. This patient recovered without demonstrable residua. The question whether *Toxoplasma* caused the actual clinical manifestations in this case must be left open.

Finally, a *Toxoplasma* infection in man can also have a subclinical course without giving any demonstrable objective symptoms. Infection can then only be proved by the presence of antibodies against *Toxoplasma* in the blood serum. This form of the disease has been demonstrated relatively often in adults as, for example, in the majority of mothers of children suffering from congenital infection. It also occurs, however, in children. Adams, Horns and Eklund thus found, in the examination of a family in connexion with a typical case of toxoplasmosis, positive neutralization tests, not only in the mother, but in all of the nine siblings of the patient. None of them showed any clinical or roentgenological signs or symptoms of toxoplasmosis. Tomlinson

found toxoplasmic pseudocysts in the cerebrum and heart of a 10½-year-old negress, who had died from a sickle cell anaemia, and who had had no clinical symptoms which could be attributed to a Toxoplasma infection.

Material.

Cases 1 and 2.

T. L., a boy, b. 23.9.1945. The father was 28, the mother 20 years of age. Both parents and the patient's sister (b. 1944) had always been healthy. The pregnancy had been normal, and delivery was normal and at term. Weight at birth 2620 g., length 46 cm. 2mg. vitamin K were given orally at birth.

On the sixth day of life the infant had a haematemesis and was therefore admitted to the Sachs Hospital for Children. An examination made on September 28th showed that the patient was afebrile and the general condition good. With the exception of slight microphthalmia, he was well-formed, normally well-nourished and had a good colour. The tonus and turgor showed no abnormalities, but his cry was somewhat weak. The anterior fontanelle was of normal width, neither tense nor bulging. The greatest circumference of the head was 33 cm., and there was no separation of the sutures. No pathological changes were found on neurological examination. Heart and lungs showed no abnormalities. The liver and spleen were palpable. The spleen reached to just below the level of the umbilicus and the liver two fingerbreadths below the arcus in the mamillary line. Roentgenological control showed, however, no demonstrable enlargement of either organ. On the anterior aspect of the trunk and the medial sides of the thighs was a small dotted haemorrhagic exanthema, which faded after a few days. Thrombocyte counts made for this reason showed low values on a few occasions. The haematemesis was shown to be due to excessive hypoprothrombinaemia. This ceased immediately after parenteral administration of vitamin K, and the prothrombin index subsequently remained within the normal limits. Blood-counts made on the 6th and 12th day of life showed a relatively large number of eosinophil granulocytes, but otherwise nothing abnormal.

The patient has since been kept under regular control and now shows the following syndrome: porencephalia and internal hydrocephalus; xanthochromia, with slight increase of cells and high protein concentration and intraventricular calcifications corresponding to the course of the choroid plexus. Attacks of general convulsions have occurred during the last two months. Bilateral microphthalmia and secondary cataract. Mental deficiency.

During the time of observation up to now, physical development has been normal. At the age of 1½ years his weight was 12½ kg. and length 77 cm. Before he was three months old it was apparent that mental development was considerably retarded. This has been increasingly evident and at times the child has been extremely lethargic. Except during transient minor infections, he has been afebrile. With the exception of the above-mentioned haemorrhagic exanthema, he has shown no cutaneous symptoms and there has been no pathological enlargement of the lymphatic glands. During the first six months of life, both the liver and the spleen were palpable, as a rule 2–3 fingerbreadths below the arcus. Neurological examinations gave only negative results. It is, however, true that at the age of 4–5 months, the patient sometimes lay with his head hyper-extended. There was, however, no neck rigidity and both the Kernig and Brudzinski signs were negative. On one or two occasions the patellar and Achilles reflexes were brisk, but nevertheless not definitely pathological. Once or twice at the age of about 1½ years he had short attacks of general convulsions. The E. S. R. has varied between 6 and

10 mm/hr. A blood Wassermann reaction at four months was negative. At the same time sternal, liver and spleen punctures were made, and a tibia puncture was taken at one year, but no pathological changes could be shown with certainty. Tuberculin tests were made regularly and gave negative results, as did analyses of the urine and faeces.

Toxoplasmosis was diagnosed clinically in January 1946, when the patient was 3½ months old. It is probable that the diagnosis could have been made considerably earlier if roentgenological examination of the skull had been made. In order to confirm the diagnosis, further examinations are, however, necessary. It can be made probable by demonstration of the presence of organisms with the morphology of *Toxoplasma* in material from the body fluids and tissues obtained at biopsy or section. Specific diagnosis can be made by isolation of *Toxoplasma* in suitably selected laboratory animals and by the demonstration of specific antibodies in the serum of the patient.

Co-operating with Dr. Gard, during the first half of 1946 repeated inoculations on experimental animals were made with various puncture biopsies from this patient. The results were all negative which could, to a certain extent, be expected since *Toxoplasma* is an intracellular parasite.

The possibility which remained, in order to confirm the clinical diagnosis, was the serological demonstration of specific antibodies in the blood serum of the child, since serum from human beings who have contracted the disease contains specific antibodies. These have, in a large number of cases, been shown to remain for many years. On the basis of this fact, Sabin and co-workers have worked out a diagnostically important method. It is necessary, in order to perform this test, to possess *Toxoplasma*. The L. M. strain of *Toxoplasma* described by Paige, Cowen and Wolf was used by us for the serological tests, which were all carried out by Dr. Gard.

A serological test was made for the first time on 19. 2. 47. and gave positive results both for the patient and his mother. On 10. 3. 47. both were tested again and specific antibodies could still be demonstrated. At the same time the father and the 3-year-old sister of the patient were tested. The results were negative in both cases. The number of reactive infection doses which were neutralized by the various sera can be seen in the following table:

	Date	Neutralized reactive doses
Patient	19. 2. 1947	125
Mother	19. 2. 1947	25
Patient	10. 3. 1947	125
Mother	10. 3. 1947	125
Father	10. 3. 1947	0—1
Sister	10. 3. 1947	1—5

The clinical diagnosis of toxoplasmosis in our patient was thus verified serologically.

The mother (Case 2) stated that she had always been healthy, but that she had suffered from intense fatigue during the last two months of the pregnancy in question, as well as during approximately four months after delivery. On account of the illness of her child, she underwent a thorough examination with negative results. She was, nevertheless, a carrier of the infection and infected her child in utero. Despite careful investigation, it has not been possible to demonstrate the source of the mother's infection.

On the basis of this first case of the disease, of which a detailed report was published earlier (Magnusson, 1947) we have, during recent months, traced and investigated further cases, in which toxoplasmosis could be suspected on various grounds. An account is given below of those cases in which the serological examinations showed positive results.

Case 3.

P.B., a boy, b. 1933, admitted to Tomtebodas Institute for the Blind. Development during the first years of childhood appears to have been normal and no symptoms of disease were observed. Eye disease was first observed in the second year of life; since then there has been increasing deterioration of sight. He attended an ordinary school for $1\frac{1}{2}$ years and was admitted to a school for the blind at the age of 9. Aqueous flare, posterior synechiae and vitreous opacities were then observed; in the left eye choroidal foci were seen. General examination revealed nothing of significance. Mantoux 1 mg. and W.R. were negative. Later, retinitis proliferans and increasing cataract appeared.

In March 1947 the patient was examined with a view to a toxoplasmic infection as a possible cause of his disease. His general condition was good. Physical development was normal, but there was some retarded mental development, probably owing to blindness. Internal organs: N.A.D.¹⁾ No cerebral symptoms could be demonstrated (encephalography was not performed). Roentgenological examination of the skull showed a streaky formation of almost chalk-like density corresponding to the left trigone. Ophthalmologically there was bilateral amaurosis with total cataract, posterior synechia, etc., thus the picture of a past, severe chronic uveitis. Serological testing was made on 21.3.47. and gave a positive result (25 neutr. reactive doses).

The case thus shows severe ocular symptoms, intracranial calcification and a positive serological reaction for *Toxoplasma*.

Case 4.

W.L., a boy, b. 1937. Admitted to Tomtebodas Institute for the Blind.²⁾ Since very early childhood he showed deterioration of sight. Already at the age of two, complicated bilateral cataract was found.

In March 1947, the patient was examined for suspected toxoplasmosis. His general condition was satisfactory and his physical development normal.

¹⁾ N.A.D. = No appreciable disease.

²⁾ It has been possible to examine Cases 3 and 4 thanks to the kind co-operation of Dr. O. Olson, ophthalmologist at Tomtebodas Institute for the Blind.

Mentally he was, however, somewhat retarded, but not more than could reasonably be attributed to blindness. Internal organs: N. A. D. Neurological examination gave negative results (encephalography was not performed). Roentgenological examination of the skull was negative. Phthisis bulbi and amaurosis were found in the right eye. There was some perception of light in the left eye, some aqueous flare, a number of synechiae and total cataract. Serological testing on 21.3.47. gave positive results (5—25 neutr. react. doses).

This case thus shows severe ocular symptoms in the form of chronic uveitis and cataract, as well as a positive serological reaction for *Toxoplasma*.

Cases 5 and 6.

I., a girl, (Case 5) b. 8.2.47. two weeks before term. The father was 26 and the mother 24 years of age. Both were previously healthy.

The mother (Case 6) was a primipara and had been under regular control during pregnancy at a maternity centre, when no pathological symptoms were observed. She was admitted to the Maternity Department on 8.2.47. and instrumental delivery was performed on the indication of threatened foetal asphyxia. At first, the pulsations in the umbilical cord were good. The infant opened its eyes once, but did not cry, and the cord was therefore severed at once. Attempts at resuscitation (warm baths, artificial respiration, »eufodrina«, lobeline and oxygen) were made, and quantities of mucus were aspirated from the respiratory tract. The infant drew a few breaths, but died a short time after birth.

The placenta and membranes were expelled without difficulty. The placenta showed a few small infarctions but otherwise nothing abnormal. The mother was relatively well after parturition. She had secondary anaemia with 53 % Hb., but otherwise showed no symptoms. Later, intensive mental fatigue occurred, which led to lack of ability to work for five months after parturition.

Autopsy (F. Wahlgren).

Nearly full-term child. Weight: 2850 g., length: 48 cm. Head very deformed with a large swelling to the left over the brow. Anterior and posterior fontanelles considerably larger than normal and all sutures in the skull separated to just over 1 cm. in breadth. Anterior fontanelle open anteriorly over the temples. Forceps marks over the nose and upper lip.

No haemorrhage between the dura and the cerebrum. Tentorium cerebelli uninjured. Practically all the cerebrum reduced to a thin flabby shell, surrounding the considerably dilated lateral ventricles. Numerous yellowish-grey opaque foci, up to the size of a pea in the cerebral parenchyma. Greyish-white, slightly cloudy fluid present in the ventricles. No visible changes in the cerebellum, pons varoli or medulla oblongata. No visible changes in the heart or the great vessels. Lungs completely atelectatic with tough engorged parenchyma. No abnormal content in the trachea or bronchi. Stomach and intestines: N. A. D. Liver enlarged and firm with dark-brown cut-surface, showing no pattern. Bile ducts and pancreas: N. A. D. Spleen moderately enlarged and of firm consistency, cut-surface dark-red and even. Adrenals and kidneys of normal size and without changes.

Microscopical examination:

Cerebrum: Within large areas of the hemispheres of the cerebrum the tissue is reduced to a layer scarcely more than one mm. thick. The tissue is in great extension necrotic and the necrotic areas are more or less

extensively permeated with finely grained calcium deposits, fat-containing cells and inflammatory cells, chiefly leucocytes. Lymphocytes and plasma cells are, however, present. The vessels are extremely dilated and engorged. In several places intracellularly situated *Toxoplasma* pseudocysts. The leptomeninges are also considerably infiltrated with inflammatory cells.

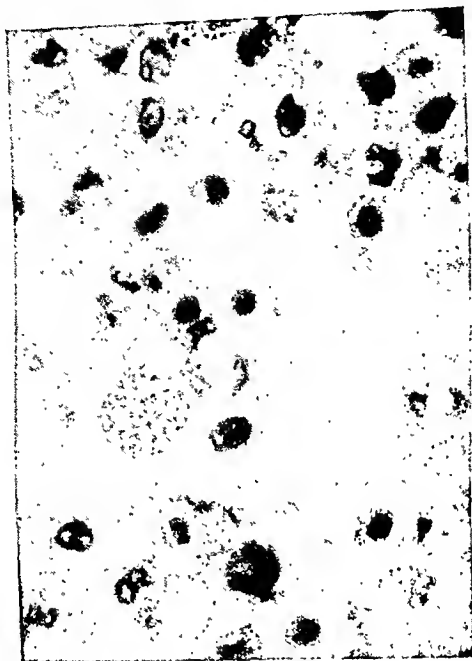


Fig. 4.

Case 5. Intracellularly situated *Toxoplasma* (pseudocyst) from cerebrum. Magnification ca. 1000 X.

In sections from the central ganglia as well, granulation tissue is found, particularly towards the lateral ventricles. This is largely necrotic and more or less abundantly permeated with calcium deposits. In the other areas the cerebral tissues show considerable inflammatory changes, are soft and full of fat-containing phagocytes and inflammatory cells, chiefly leucocytes. The vessels are very engorged and in several places there are fresh haemorrhages.

Liver: The acinic patterns are somewhat indistinct. The parenchymal liver cells show no remarkable changes. The intra-acinous capillaries are dilated and contain a considerable amount of red corpuscles. In parts of them there are also agglomerations of nucleus-bearing red corpuscles. The Kupffer cells do not appear to be swollen. In the periportal connective tissue, here and there small clumps of lymphocyte-like mononuclear cells. Biliary tract and vessels: N. A. D. *Toxoplasma* was not demonstrated.

Spleen: The follicles are relatively large and ill-defined. They contain no growth centres. The splenic pulp is moderately engorged. No swelling of the pulp cells or of the sinus endothelial cells can be demonstrated, nor are any pathological cell elements found. *Toxoplasma* was not demonstrated.

Kidneys: Show no noticeable changes.

Adrenals: The cortex is mainly normal in appearance. Hyperaemia is observed within the medulla, and in parts small fresh haemorrhages. No

necroses. Toxoplasma was not demonstrated. Spirochaetae were not demonstrated in slides stained according to Levaditi of the liver, spleen, adrenals and kidneys.

Pathological diagnosis: Toxoplasmosis + hydrocephalus internus + hyperplasia hepatis et lienis.

Since the child thus showed pronounced tissue injuries, typical of toxoplasmosis the mother was tested serologically. This test was made on 28. 3. 47. and gave a positive result (125 neutr. reactive doses). The diagnosis of toxoplasmosis was thus established in both cases. The mother, with her subclinical infection, had infected her child in utero.

Cases 7 and 8.

H., a boy, (Case 7) b. 28.11.45. two months before term. Weight at birth 1160 g. The father was 36 and the mother 37 years of age. Both were healthy. There were healthy siblings, aged 7 and 4. Nothing of interest in the hereditary history.

The pregnancy had a normal course, with the exception of the last two weeks, when uterine haemorrhages occurred. Nothing abnormal occurred in connexion with delivery. The child was born in a cottage hospital in the country and was only admitted to a children's hospital after a few days on the grounds of prematurity. He was treated there from 5.12.45. to 20.3.46. with the diagnosis of debilitas congenita + hypoprothrombinaemia + pemphigus neonatorum. The following extracts from the journal can be noted: On admission the temperature was only 33.5° C. The patient was, however, fairly lively and cried strongly. He was thin and the body covering was flaccid. He weighed 960 g. Length 35 cm., circumference of the head 26 cm. The skin was thin and easily detached and showed considerable lanugo, particularly on the shoulders. The skin was of a normal pink colour. On the right cheek, neck and chest, as well as on the medial aspect of the left thigh, there were pea-sized pemphigus vesicles, as well as remains of others. Umbilicus: N. A. D. No cyanosis or oedema. The skull showed nothing abnormal. Throat and oral cavity: N. A. D. Respiration was rather slow, pulmonary findings normal. The anterior fontanella was 1 × 1 cm., neither tense nor bulging. The cardiac sounds were clear and regular with a frequency of 110/m. The liver and spleen were not palpable. Neurologically, nothing pathological. There were no testicles in the scrotum, the right testicle was palpable in the inguinal canal.

Blood counts taken on admission showed > 140 Hb. The leucocyte count was 12,100, of which 5,700 were polynuclears. Hb. estimation decreased to a minimum value of 70 % at six weeks, but subsequently was just over 80 %.

The child developed satisfactorily during his stay in hospital. He showed no rise in temperature beyond the usual fluctuations in premature infants. He received breast milk with Aminosol glucose as supplementary nourishment during the first three months and thereafter citrated milk. He was given iron, and vitamins A and D prophylactically. On discharge from hospital (at a little over the age of 3½ months) his weight was 3290 g. Subsequently, at home, the parents noticed that in comparison with their two older children, his development was retarded. He could not focus and, according to their statements, »rolled his eyes here and there and anyhow«. It was only at the end of May 1946 that the eye movements improved. Somewhat later it was noticed that the pupils were grey, and since he was still unable to focus, an ophthalmologist (Dr. Granström) was consulted in January

1947. Examination showed the following: Inability to focus, the eyes not injected. The right cornea somewhat opaque, the left almost clear. Anterior chamber almost deficient. On the right, some posterior synechia, widespread in the left. Left iris atrophic; no definite pupil reaction; the lens bulging forward, the right partly, the left totally opaque. No red reflex. Pressure normal. A toxoplasmic infection was suspected as a cause of the ocular symptoms.

The patient was admitted on 16.4.47, to the Saelis Hospital for Children. He was then just over 16 months of age. He was pale and thin. Both tonus and turgor were diminished. Weight: 7600 g., length: 75 cm., and circumference of the head 44 cm. Skull firm and normal in appearance; anterior fontanelle was closed. No symptoms of rickets. Temperature normal. No positive pathological findings in the internal organs. E. S. R. 12 mm/hr. Hb. 80 %. Urine: N. A. D. Roentgenological examination of the skull gave negative results (encephalography was not performed). Marked mental deficiency. General behaviour characterized by uneasiness and crying and he had no contact with his surroundings. Sat unsteadily and only for a short time. Unable to raise himself to a sitting position. Did not stretch after objects, could not stand. Eye findings entirely unchanged. Serological tests made on 25.4.47. gave positive results (25—125 neutr. reactive doses).

The positive serological reaction for *Toxoplasma*, together with the ocular symptoms and mental deficiency, confirm the diagnosis of toxoplasmosis in this case. The time for the appearance of the symptoms shows that the infection was congenital.

The mother (Case 8), who had previously been healthy, and even then showed no symptoms of illness, was tested serologically at the same time as the child. The test was also positive (25 neutr. react. doses). The *Toxoplasma* infection probably had a subclinical course in her case. During the pregnancy in question, she was a carrier of the infection, and infected her foetus.

Cases 9 and 10.

Lc., a boy, (Case 9) b. 25.11.37. approximately two weeks before term. The father was 32 and the mother 28 years of age. Both, with the exception of minor infections, had been healthy.

The mother (Case 10) was a primipara and had been under regular control during pregnancy. During the last two months of pregnancy she had been mentally extremely tired and suffered from slight anaemia. She was admitted to the Maternity Department on 24.11.37. and the child was born on the following day after a normal parturition.

The placenta and membranes were expelled normally. The placenta was whole, and showed no abnormalities. The mother was relatively comfortable after delivery. She was afebrile during the whole time, but was slightly anaemic and extremely tired. This fatigue, which subsequently increased somewhat, made her unfit for any work for 3—4 months after delivery.

The infant's colour was good at first, respiration was good, but he cried weakly. After half an hour his colour became extremely cyanotic and respiration very shallow. Despite all therapeutic measures, his condition did not improve and he died after two hours of life.

The ocular symptoms in Cases 1, 3, 4 and 7 will be the subject of a special paper by K. O. Granström and J. H. Magnusson.

Autopsy (F. Wahlgren).

Nearly full-term infant. Weight: 2630 g. Length: 49 cm. Head strikingly small — circumference 29.5 cm — and the cervical region in particular very under-developed. Otherwise nothing unusual observed on external examination. Between the dura and the cerebrum a clear, brown fluid. A thin coating of yellowish-brown membrane on the inner aspect of the dura.

Cerebrum collapsed, small and flabby. Considerable quantities of yellowish-brown cloudy fluid contained within the leptomeninges. Cerebral parenchyma considerably changed, but could not be examined more closely until the cerebrum had been fixed. Heart and great vessels: N. A. D. Lungs practically entirely air-filled with no changes. No free fluid in the abdominal cavity. Liver somewhat enlarged and firm in consistency. Cut surface yellowish-brown, without pattern. Spleen considerably enlarged and firm in consistency. Cut surface reddish-brown. Adrenals, kidneys and urinary tract: N. A. D. At lower end of femur, demarcation between bone and cartilage sharply defined, with cartilage remarkably easily detached from the bone. No visible changes in the stomach and intestines.

Microscopical examination of preserved tissues, carried out in 1947.

Only parts of the cerebrum and of the other organs mentioned below are preserved, embedded in paraffin.

The cerebral parts are from the cerebrum and extend into the ventricular system, so that the ependyma and parts of the choroid plexus are also found. In large areas the cerebral tissue is entirely destroyed and plentifully infiltrated with finely-grained calcium deposits. In the other parts the tissue is soft and considerably permeated with fat-containing phagocytes as well as inflammatory cells. These consist partly of leucocytes and partly of plasma cells and lymphocytes. The ependyma is fairly well preserved. The vessels are much dilated and engorged. Here and there are small fresh haemorrhages.

Toxoplasma localized intracellularly (pseudocysts) were demonstrated in one place only.

Parts of the leptomeninges are also included in the sections. They are very abundantly infiltrated with inflammatory cells, of the type mentioned above. The vessels are much engorged.

Liver: The acinic pattern is indistinct. The parenchymal cells show no marked changes. Small vesicles, however, appear in places in the protoplasm, indicating a certain degree of fatty degeneration. The intra-acinary capillaries are somewhat wide and contain red corpuscles — of which many are nucleus-bearing — as well as leucocytes and lymphocytes. The Kupffer cells are often swollen and sometimes show a picture of phagocytosis. Large groups of cells, of myeloblastic and myelocytic type, are frequently seen in the periportal connective tissue, as well as more or less mature leucocytes and occasional lymphocytes. The bile duct and biliary vessels show no evident changes. No necroses were demonstrated, nor were Toxoplasma observed.

Sections from the cartilage-bone junction at the upper end of the tibia show fairly normal bone marrow and no evident changes within the bone-forming zone.

Spleen: The follicles are indistinct and ill-defined against the pulp. They contain no growth centres. The pulp is moderately engorged and fairly poor in cells. Apart from nucleus-bearing red corpuscles, no pathological cell elements appear to be present. No Toxoplasma were demonstrated.

Kidneys: In the stroma of the cortex small groups of lymphocyte-like cells are seen in a few places. Otherwise the renal parenchyma shows no notable changes. No Toxoplasma were demonstrated.

Spirochaetae were not demonstrated in the Levaditi-stained slides of the liver, spleen and kidneys.

Pathological diagnosis: Toxoplasmosis + erythroblastosis.

The mother was tested serologically on 25. 4. 47. This test gave a positive result (> 25 neutr. react. doses) and the diagnosis of toxoplasmosis was confirmed in both cases. The mother had been a carrier of the infection and had infected her child in utero.

It is of interest to note that the mother, in 1939 and 1942 — thus $1\frac{1}{2}$ and 5 years respectively after the birth of the child with toxoplasmosis — gave birth to two healthy children. At a recent examination, both these children were clinically free from symptoms.

Cases 11 and 12.

E., a girl, (Case 11) b. 1.10.34, two months before term. The father was 26 years old and healthy. The mother, who since the age of 17 had suffered from a slight cardiac disorder, but was otherwise healthy, was 25.

The mother (Case 12) had a miscarriage in 1929. During the pregnancy in question here she had only been sporadically controlled, but on these occasions showed no pathological symptoms. During the last weeks of pregnancy she felt »very tired and peculiar« and towards the end the abdomen was extremely swollen. On 20.9.29, labour pains started, but ceased after a short time, but there was considerable escape of the liquor amnii. On the evening of 30.9.29, the labour pains started once more, and the foetus was born on the following morning after a normal delivery.

The placenta and the membranes were expelled spontaneously. The placenta was whole and showed nothing abnormal. Following delivery, the mother had a slight rise in temperature (resorption fever) for a few days, and felt increasingly tired. This discomfort subsequently increased to such an extent that she was unable to work for about 8 months after delivery.

During May/August 1934, the mother worked as a domestic servant in a family in the country. In her duties was included the care of a fox-terrier, who — according to her statements — had numerous ticks in its fur. She found ticks on her own skin very often during this time. The dog died in 1935, but no investigation into the cause of death was made.

The infant was extremely oedematous, only breathed once or twice, and died after one minute.

Autopsy (F. Wahlgren).

Full-term infant, colour of skin pale reddish-brown. Length: 48 cm. Weight: 3120 g. Circumference of head: 37.5 cm. No signs of maceration. Massive oedema in the subcutaneous tissue 1 cm. thick, for example, in the abdomen. On pressure exuded plentiful quantities of yellow, clear fluid.

A suggestion of hydrocephalic shape in the skull, with wide sutures. Cerebrum consisted of a flabby, very thin-walled sack, extremely flaccid and soft in consistency. Ventricle system greatly widened, containing a clear, yellowish-brown liquid with fairly plentiful granulated yellowish-brown sediment. Cerebral tissue also yellowish-brown in colour. In the hemispheres of the cerebrum no visible difference between the cortex and the medulla. Nothing of special interest in the dura or dural sinuses. No intracranial haemorrhage. The hypophysis cerebri and the thyroid of normal size, without macroscopic

changes. Thymus smaller than usual, flaccid and tough in consistency, cut surface reddish-grey. No malformations or other changes in the heart. Great vessels: N. A. D.

About two tablespoonfuls of reddish-yellow clear fluid in each pleura. Pulmonary parenchyma greyish-white, nowhere air-containing. Mucosa of the trachea and bronchi pale. About 100 cc. of yellowish-red clear fluid in the abdominal cavity. *Stomach and intestines* without gas. Liver of normal size and firm consistency. Cut surface brown, without pattern. Bile ducts, pancreas and portal vein: N. A. D. *Spleen* very considerably enlarged, about 5—6 times normal size. Firm in consistency. Cut surface reddish-brown and smooth. *Adrenals* of normal size, cortex fairly thin and yellowish-brown. Medulla reddish-brown and moist. *Kidneys and urinary tract*: N. A. D. Umbilical vessels: N. A. D.

At the lower end of the femur, the border of the epiphysis marked as a yellowish-brown, somewhat irregular streak, considerably broader than usual.

Microscopical examination of preserved parts of tissues, carried out in 1947.

The examination material consisted of paraffin-embedded parts of the cerebral hemispheres and the central ganglia.

The cerebral tissue is completely necrotic in very large areas and more or less plentifully infiltrated with calcium deposits. There are also several fresh haemorrhages. Between the above-mentioned areas, which principally appear to consist of the areas nearest to the ventricular system, there are parts where the cerebral tissue is more or less colliquative and often plentifully infiltrated with inflammatory cells. The majority of these consist of leucocytes, of which many are eosinophils. There are also lymphocytes and plasma cells, as well as numerous fat-containing phagocytes. The vessels are considerably dilated and engorged. The arachnoidea is plentifully infiltrated with inflammatory cells of the kind just mentioned. Intracellular agglomerations of *Toxoplasma* are found in several places.

Liver: The acinic pattern only appears indistinctly. The parenchymal cells in the central parts of the acini are often entirely destroyed and a narrow strip of more or less degenerated parenchymal cells is found only in the periphery of the acini. The central parts of the acini are often plentifully infiltrated with small lymphocyte-like cells — single leucocytes and normoblasts and myeloblasts. The cells of the reticulum appear to be fairly well preserved. The intra-acinary capillaries are wide and contain nucleus-bearing red corpuscles. The periportal connective tissue is not more abundant than usual, but it contains abundant agglomerations of mononuclear lymphocyte-like cells, as well as cells of myelocyte and myeloblast type. Bile ducts and vessels: N. A. D. No *Toxoplasma* demonstrated.

Spleen: The follicles are small and indistinctly defined against the pulp, which is engorged and poor in cells. Precursors of red and white corpuscles are found in the pulp. No swelling of the actual pulp cells. No *Toxoplasma* demonstrated.

Kidneys: The interstitial tissue, both within the cortex and the medulla, is very plentifully infiltrated with lymphocytes and precursors of red and white corpuscles. The tubules are ruptured by this great agglomeration of cells. Neither the epithelial cells of the tubules nor the glomeruli show any marked changes.

The perirenal fatty tissue is particularly abundantly infiltrated with precursors of red and white corpuscles. No *Toxoplasma* demonstrated.

Adrenals: The columns of cells appear fairly clearly within the cortex and the cells of the cortex show no marked changes. The medullary tissue of the marrow is particularly abundantly infiltrated with precursors of red and white corpuscles. There are also recent haemorrhages. No *Toxoplasma* demonstrated.



Fig. 2.

Case 11. Intracellularly situated *Toxoplasma* (pseudocyst) from cerebrum.
Magnification ca. 1000 X.

Bone-marrow: Sections from the cartilage-bone junction at the upper end of the tibia show no marked abnormalities within the bone-forming zone.
Bone-marrow: N. A. D.

Spirochaetae were not demonstrated in Levditi-stained slides from the liver, spleen, adrenals or kidneys.

Pathological diagnosis: *Toxoplasmosis* + erythroblastosis.

As a result of the observations made on fresh examination of the anatomical preparation from this child, the mother was tested serologically. The test made on 9. 5. 47. gave a positive result (25 neutr. react. doses), and showed that toxoplasmosis had been present in both cases. The infection had been transmitted to the foetus by the mother, who was suffering from a subclinical infection.

In 1936 and 1937, thus 2 and 3 years respectively after the birth of the child with toxoplasmosis, the mother gave birth to two healthy children. They were recently examined, together with their mother, and were found to be healthy.

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I.
cases of toxoplasmosis in the material.

Serological examination	Pathological changes	Diagnosis	Place and date	Remarks
+	—	Clinical Serological	Stockholm 1945	Blind, mental deficiency. Birth weight: 2620 g. Hypoprothrombinaemia.
+	—	Serological	Stockholm 1945	Intense fatigue 2 mths. before and 4 mths. after delivery. No other symptoms. Gave birth to healthy child 1944.
+	—	Clinical Serological	1934—1935	Blind.
+	—	Clinical Serological	1937—1938	Blind.
Not performed	Changes typical of toxoplasmosis in the cerebrum, with intracellular Toxoplasma	Autopsy	Stockholm 1947	Birth weight: 2850 g.
+	—	Serological	Stockholm 1946	Intense mental fatigue. Entirely unable to work for 4 mths. after delivery.
+	—	Clinical Serological	Vagnhärad 1945—46	Blind. Mental deficiency. Birth weight: 1160 g. Hypoprothrombinaemia.
+	—	Serological	Vagnhärad 1945	No symptoms of disease. Two healthy children born 1938 and 1941.
Not performed	Changes typical of toxoplasmosis in the cerebrum, with intracellular Toxoplasma	Autopsy	Stockholm 1937	Asphyxia and marked cyanosis. Birth weight: 2630 g. Erythroblastosis.
+	—	Serological	Stockholm 1937	Anaemia and considerable mental fatigue 2 mths. before and 3—4 mths. after delivery. No other symptoms. Bore two healthy children 1939 and 1942.
Not performed	Changes typical of toxoplasmosis in the cerebrum with intracellular Toxoplasma	Autopsy	Stockholm 1934	Birth weight: 3120 g., born 2 mths. before term. Congenital hydrops. Erythroblastosis.
+	—	Serological	Stockholm 1934	During the weeks immediately prior to delivery »tired and peculiar«. After delivery severe mental fatigue for 8 months. Bore two healthy children 1936 and 1937.

Discussion.

The diagnosis of toxoplasmosis can be considered definitely confirmed in the cases described here. As mentioned earlier, this diagnosis can not be made with certainty on the grounds of the clinical manifestations alone, or by demonstration of organisms of Toxoplasma morphology. The demonstration of specific antibodies, or the isolation of Toxoplasma by means of inoculation of animals, is required for a more specific diagnosis. Nine cases (Nos. 1, 2, 3, 4, 6, 7, 8, 10 and 12) were tested serologically, and in all of these it was possible to confirm the diagnosis by the demonstration of specific antibodies. In the remaining three cases (Nos. 5, 9 and 11), such tests could not be made. In these cases there was, however, a typical pathological picture, and organisms of Toxoplasma morphology were demonstrated in each case. In further support of the accuracy of the pathological diagnosis, we have the fact that the mothers of these three infants who died immediately after birth were serologically positive.

These twelve cases of toxoplasmosis are the first to be diagnosed in Sweden. As is seen from the material, this infectious disease is not, however, new in our country. Case 11, for example, occurred as early as 1934.

Despite careful investigation, it was not possible to show the source of infection in any of the cases. Only in Case 12 did we obtain information of the exposure to ticks.

Summary.

After a short historical survey of human toxoplasmosis, an account is given of twelve cases of the disease in Sweden, in which the diagnosis could be made with certainty.

The material can be divided as follows:

- 1) Five cases of congenital toxoplasmosis.
- 2) Two cases with residual symptoms after an earlier probable congenital Toxoplasma infection.
- 3) Five cases of subclinical infection in mothers of children with congenital toxoplasmosis.

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DISCUSSION

S. WINBLAD: In December 1946, in the Flenburgska Childrens' Hospital in Malmö a case of toxoplasmosis was observed, in which the diagnosis, however, was established post mortem.

This was the case of a newborn girl, who was admitted for observation already shortly after birth. Even at delivery a pronounced enlargement of the spleen and liver could be made out. There was slight icterus. The placenta had been enlarged. Blood-grouping of the child as well as the parents gave no evidence of erythroblastosis. The microsedimentation rate was early increased to 6 mm./hour. The blood picture and temperature were normal. The brain was distinctly affected, the child being drowsy all the time. Gradually the temperature became irregular, and penicillin was employed for some length of time without any noticeable effect. Two abscesses were noticed in the occipital region, and cultures from them yielded growth of *staphylococcus aureus*, on which account sepsis was looked upon as the most probable diagnosis. After two months of illness, the child died.

Autopsy revealed pronounced changes in the brain. Here the ventricles were distinctly dilated, and large parts of the central regions of the brain facing the ventricles had undergone necrosis. Areas of calcification were numerous within these necrotic structures. These changes were found only within the cerebrum, whereas cerebellum and the medulla oblongata were normal. The liver had undergone pronounced changes reminding of cirrhosis; it was greenish in colour. There was also splenomegaly (weight 82.5 g.). The lungs showed terminal capillary bronchitis. Otherwise the organs showed no abnormality.

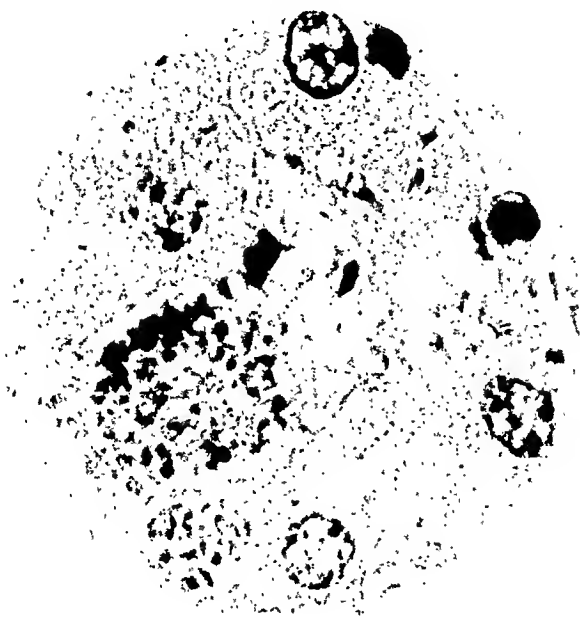
On microscopic examinations the liver was found to be studded with small necrotic foci of inflammatory character. It had a certain resemblance to congenital syphilis. Sections from the brain were more suggestive of necrosis with small areas of calcification than of inflammatory processes.

Thus the picture presented by the autopsy findings seemed indicative of chronic sepsis together with cirrhosis of the liver. Still the Wassermann test had been performed on the mother repeatedly and always turned out negative so that any suspicion of congenital syphilis found no support in this way. Before long, however, the idea of congenital toxoplasmosis suggested itself when we learned that such cases had occurred elsewhere in Sweden — and also in Switzerland. So far, however, the diagnosis could not be confirmed histologically by demonstration of toxoplasma. Through the kind collaboration of Docent Gard the mother's blood has been examined serologically and in this way it has been ascertained that even though the serum had been left standing for a rather long time, it still was able to neutralize 5 reactive doses.

In view of the autopsy findings — necrosis with calcification of the brain and miliary necrosis of the liver — together with the po-

sitively serological findings in the mother's blood, we here appear to have been faced by a case of congenital toxoplasmosis. So it may be of considerable interest in the future to pay close attention to such cases as perhaps they are not so exceptional, but occurring in our countries now and then.

S. RUBARTH (*Presented by H.-J. Hansen*): The material of diseased game animals examined by Professor Hülphers, The Veterinary High School, includes several hares showing a uniform autopsy picture in which no bacteriological examination or inoculation of normal animals with material from the hares yielded any evidence of infection. Nor did toxicological examination clear up the cause of death. In order



Liver cells with Toxoplasma.

to obtain a more thorough pathologic-anatomical estimation of this material, it was sent to me in the hope that my examination of it possibly would yield some evidence of virus infection.

The total number of the hares in question was 27, all of which had been found dead in wintertime in southern and middle Sweden. Usually the animals were found in open fields, giving the impression that death had come quickly, without any preceding severe illness.

On an average, autopsy revealed a state of good nutrition. The lungs, liver and spleen were hyperemic, and the body cavities especially the pleural, contained some serous transudate, often with an admix-

ture of blood. In most of the cases the liver was studded with foci, light in colour and varying in size, while the mesenteric regional lymph glands largely had undergone necrosis.

Histological examination of the liver showed often numerous areas of necrosis without any definite localization in the lobules. Usually the necrotic areas were sharply defined, without any surrounding reaction, and presenting a distinct reticular structure, due to precipitation of fibrin. In the mesenteric lymph glands the necrosis was always of central location, often involving the entire gland — with exception of a narrow border of intact lymphoglandular tissue adjacent to the marginal sinus. The transition from the necrotic part of the gland to the intact tissue was indistinct. Often the surrounding connective tissue was the site the of inflammatory changes.

In most of the cases also the myocardium, and sometimes the brain, were also examined histologically — without presenting any particular abnormality.

In all the cases, round congregations of toxoplasma could be demonstrated in the cells of the liver as well as in endothelial cells without any direct connection with the necrotic processes described above. The parasites were lying in pseudocysts closely adjacent to apparently intact cells. In the border of necrotic changes surrounding the necrotic processes in the lymph glands, strikingly many intracellular accumulations of toxoplasma were encountered, and the same applies to the inflammatory connective tissues surrounding the lymph glands. The above described localization of the toxiplasma in the liver is suggestive of a fairly old infection, possibly contracted in the summer. Weinmann has observed such chronic infections in experimental animals, and I have seen the same morbid picture in a dog that had been given an experimental infection, 3 months before, and now died of an intercurrent disease.

SOME ASPECTS OF THE NORMAL, ANTI- BACTERIAL DÉFENCE

By O. Maaaloc.

In this paper some results from studies of the mechanism of bacterial infections will be compared with theoretics and results from coagulation- and complement-research. For experimental data, references will be made to other publications.

It is necessary first to limit the subject; and in order to obtain relatively simple conditions we shall, in the following, deal only with the acute phase of bacterial infections, i. e., all immunological phenomena are left out of consideration. Furthermore, we shall limit our studies to a special case: the *Salmonella typhimurium* infection in mice.*)

The course of this infection is well known from earlier works by Ørskov, Jensen & others (1, 2). The essential feature is that pathogenic strains of this bacterium are able not only to invade the organism through the intestinal wall, but that they resist the antibacterial forces well and, therefore, rapidly multiply inside the organism. In the end bacteria swarm over the organism, and, though the individual bacterium is but slightly toxic, the animal dies from a general intoxication. This final phase is reached when the amount of bacteria, constantly sent into the blood-stream from the infectious foci, becomes so great that even the very active phagocytes of the liver and spleen can no longer keep the bacteriaemia down.

Jensen (3) succeeded in isolating a relatively apathogenic variant of *S. typhimurium*. It was found that this strain, which does not differ from the original one in morphology or antigenic structure, is also able to penetrate the intestinal wall and create primary foci in the regional glands. When these foci have been established, the infection, however, takes a course very different from that just described; the infection

*) This limitation is the same as is used in a paper to be published shortly (5), dealing with »pathogenic-apathogenic transformation» of the same strains of *S. typhimurium* as are referred to in this paper.

does not spread much beyond the primary foci, and it is clear that the antibacterial forces acting in and around the foci are able to prevent a generalization of the infection. The foci themselves may, however persist for quite a long time. — Using the term *invasiveness* to designate the bacterium's faculty for multiplication inside the organism, we describe the two strains of *S. typhimurium* as invasive and non-invasive respectively. With this definition it is clear that an invasive bacterium is one which resists the antibacterial forces of the organism better than a non-invasive bacterium of the same type does.

Having seen how completely the course of the infection with *S. typhimurium* depends on multiplication of the bacteria in the primary foci (2), we shall now examine what antibacterial forces are acting on the bacteria in these foci. — From *in vitro* experiments it is known that *S. typhimurium* is resistant to the action of thermostable bactericidal substances in serum, but that both the original strain and its variants are susceptible to the action of complement (4). Moreover, the writer has shown that complement is the active principle in the important phagocytosis-promoting process as well as in the hemolytic and bactericidal processes (4). One must, therefore, expect complement to play an essential part in and around the bacterial foci where phagocytosis by leucocytes is the most important antibacterial action.

It is shown in another paper (5) that a striking correlation can be demonstrated between pathogenicity and resistance to complement *in vitro*: It is invariably found that the pathogenic strain, *i. e.*, the one that best resists the antibacterial forces *in vivo*, is much more resistant to complement *in vitro* than the apathogenic strain. Together with experiments showing that the two strains have nearly the same toxicity, this correlation between pathogenicity and resistance to complement is very convincing, and points directly to complement as the most active factor in the normal defence against the *S. typhimurium* infection (5).

At this point, however, we come up against a theory, put forward on different occasions, which claims that the activity of complement is zero or at least very low in the circulating blood.

In support of the early French theories concerning the origin of complement, Wollmann (6) published the following experiment: Into a closed section of the *vena jugularis* of a rabbit sensitized cholera vibrios or bird erythrocytes were injected, and at intervals samples were withdrawn for microscopical examination. Within about 10 min. from the injection no complement activity could be seen, while in a control test with serum from the same animal a strong complement effect was noticed after 4—5 min.

Later, and from quite another point of view, Fuchs studied the hemolytic activity of complement in plasma prepared from rabbits in such a way that coagulation was prevented without addition of in-

hibiting substances (7). These experiments showed that complement did not act until coagulation was started (or started spontaneously) (8,9). Based on these experiments and on several physico-chemical resemblances between prothrombin and the so-called midpiece of complement (C_1), *Fuchs* postulated identity between prothrombin and C_1 , and worked out an elaborate theory, the essence of which is that the coagulation- and complement processes are competitive with regard to prothrombin (or C_1) (8,9). This theory has met with much opposition (10, 11, 12, 13) and is now hardly taken seriously. However, refuting *Fuchs's* theoretical construction should not make us forget the experimental facts on which he based his theories; and though some of *Fuchs's* experiments, which are technically difficult, have not been successfully reproduced by others, many of his results have never been disputed: *viz.*, that prothrombin and C_1 are closely related substances (10, 11), and that the coagulation and complement processes are both inhibited by different agents, such as excess of CO_2 (14, 15), heparin and cobra venom. *Fuchs's* observation, stressing the similarity between the two processes, thus point in the same direction as *Wollmann's* results and strongly suggest that, like coagulation activity, complement activity is very low in the circulating blood.

We shall now, partly based on the writer's experiments, try to carry further the comparison between coagulation and complement activity. The following 4 points will be dealt with separately:

- A) The relation between prothrombin and C_1 .
- B) The effect of Ca-precipitating substances on the processes.
- C) The relation between Thrombokinase and the fourth component of complement (C_4).
- D) The inhibiting quality of some substances with regard to both processes.

A: The »likeness« between prothrombin and C_1 has been mentioned, and in this connection reference should be made to a survey of the problem given by *Osborn* (16). — As long as neither prothrombin nor C_1 are sufficiently well characterized chemically and physico-chemically, this »likeness« can not, as an isolated phenomenon, claim too much importance.

B: As regards citrate, oxalate and other Ca-precipitating salts, a detailed description of their effects on complement is given by the writer (4). The experiments show that a perfect parallelism exists between the inhibiting effects of the different salts on coagulation and on complement activity respectively. The conclusion that free Ca-ions are necessary for both processes, is supported by a recent work by *Heidelberger* and collaborators (17). These investigators find that serum inactivated by dialysis against a phosphate buffer can be reactivated with Mg- or Ca-salts; they find, however, that Mg has greater effect than Ca. — The fact that several earlier workers (*Fallose* (18),

Ecker & Pillemer (19) and others) have failed to register the inhibiting effect on complement of citrate and oxalate is readily explained by the dilution they interpose between the addition of the salts and the determination of the complement activity. This dilution of the citrate- or oxalate-plasma alters the equilibrium between bound and free Ca (or Mg) in favour of the latter, and such experiments, therefore show the effect not of the *original* concentration of citrate or oxalate but that of the much *lower* concentrations attained through the dilution (4).

C: As to kinase and C_4 (the complement component that can be destroyed by ammonia), several qualitative resemblances can be demonstrated: 1) both substances are, presumably, lipoproteins: 2) both substances are stable at 56°, but are destroyed rapidly at about 70° (20, 25); 3) both substances are present in serum, while in the organism kinase is found intracellularly in thrombocytes, leucocytes and other cells; the leucocytes are known also to contain C_4 (21); 4) most important in this connection is, however, *Deissler's* observation (22), that C_4 is involved in the first step of the complement process, *i. e.*, just as is the case with the kinase in the coagulation process. *Deissler's* results have been confirmed by the writer (the experiments are not published).

D: Here I shall briefly point out that most — if not all — coagulation-inhibiting substances have been found also to inhibit the action of complement. Quantitative differences are, however, met with, as in the case of heparin, where the concentration required for total inhibition of coagulation has only a minor effect on the action of complement. That the inhibitions are, nevertheless, of a like nature may be concluded from the experiments of *Regamey* (23), who has demonstrated that a determination of the inhibiting effect on hemolysis of a heparin preparation can be used directly to express its anti-coagulant potency.

Summarising the above statements we may cautiously conclude that, from several *independent* points of view, great resemblance is found between the coagulation and complement processes. We shall, of course, not go as far as to identify any pair of components taking part in the two processes; but we may safely assume the processes to be of similar nature and constitution.

That we shall not expect some day to be able to identify the two processes is clearly seen from the following signal difference between them: When kinase is added to a system containing all other coagulation components *except fibrinogen*, the conversion of prothrombin to thrombin starts immediately and is completed within 30—60 min. In fresh serum we have the prothrombin-like C_1 , the kinase-like C_4 together with Ca and Mg in the same solution and nothing seems to happen; after several hours we may still inactivate the serum by Ca- and Mg-precipitation or by destruction of C_4 . It seems, therefore, that the complement process does not start *until some object for its action is present*. — *Fuchs* was well aware of this difference, but his theo-

ries do not account for the fact that, a strong complement activity is found at a time when all prothrombin ($= C_1$ according to him) is converted to thrombin and thus, still according to *Fuchs*, rendered useless as complement component. — In a recent work, *Owren* (26) has described a new clotting-factor (factor V), and given an explanation of the mechanism of the autocatalytic process leading to the conversion of prothrombin to thrombin. It is very likely that *Owren's* theories and his refined methods for separation of the different clotting-factors will prove useful for comparisons between the coagulation and complement processes. It is tempting to look for a functional parallelism between *Owren's* thermolabile factor V and C_2 (the complement end-piece).

After this short comparison of the coagulation and complement processes we shall go back to the mechanisms of infection and antibacterial defence. — We have seen: 1) that the course of the *S. typhimurium* infection depends on the multiplication of the bacteria inside the organism, i.e., on their invasiveness; and 2) that the correlation between pathogenicity and resistance to complement *in vitro* strongly suggests that, in the present case, complement is the principal antibacterial factor; 3) that, presumably, the complement activity is very low in the *intact* organism; and 4) that the coagulation and complement processes resemble each other closely in several respects. Finally, we have noticed the important fact 5) that *primary foci have been established* before any difference is seen between infections with pathogenic and apathogenic strains.

From the points 3 and 4 we derive the following hypothesis: That the coagulation and complement processes are activated under the same conditions and in a similar way.*) — It follows from this hypothesis that in and around a bacterial focus, where thrombocytes, leucocytes and other cells are being destroyed with the liberation of kinase and C_4 , an activation of both coagulation and complement activity must be expected. That the coagulation process is activated we know from the histological picture, where clots are regularly found in the small vessels around the focus.

Conclusion: We are now led to form the following picture of the defence against an acute infection of the type studied here: The bactericidal and especially the phagocytosis-promoting action of complement, which largely determines the degree of phagocytosis by leucocytes, is the most important factor in a *latent antibacterial defence*. — I. e., a defence which is activated locally, as the coagulation process is, when, owing to mechanical or toxic cell-destruction, the necessary activating substances are liberated. We must thus view the zone immediately around a bacterial focus as constituting a barrier with high antibacterial activity through which a bacterium must pass in order to penetrate deeper into the organism and create more central

*) In principle this is *Fuch's* point of view; only, most of the experiments on which he based this part of his theories are especially difficult and have not been successfully reproduced.

foci. It is seen how this conception of the antibacterial defence mechanism reconciles the above observations 2 and 3; *i. e.*, that complement seems to be the most important factor for the defence, and that, in the *intact* organism, complement activity seems to be very low.

The results of this purely deductive work must not be taken to be more than a working hypothesis, which the writer hopes will prove useful to future investigators in this field. — Among the problems first to be solved are: Can it be experimentally confirmed that the activity of complement is negligible in the intact organism? Is there, as the writer's preliminary experiments indicate, an initial phase with especially strong complement activity just after bleeding the animal *i. e.* while coagulation is going on?

In conclusion the writer points out one practical inference of the theory: The picture given here of the normal antibacterial defence may explain why so little clinical value is attached to determinations of the antibacterial activity of sera from patients. The views put forward in this paper indicate that examinations of blood withdrawn from the organism are irrelevant if we want to determine the resistance of the patients to infections. The strength of the antibacterial forces in *serum* seems less important than the question of how rapidly and completely the *local* activation of the complement activity is brought about *in vivo*. Unfortunately, it is very difficult to attack this problem experimentally.

Summary:

The courses of infections with pathogenic and apathogenic strains of *Salmonella typhimurium* are described; and it is shown that the difference in pathogenicity between the strain is mirrored in the difference in their resistance to complement.

A survey is given of the different points of resemblance between the coagulation and complement processes.

Based on this resemblance the hypothesis is forwarded, that the two processes are activated under the same conditions and in a similar way. This implies that both processes must have very low activities in the *intact* organism.

Finally, it is described how this hypothesis leads to the conception of a *latent*, antibacterial defence, which is activated *locally* when, through cell-destruction, the necessary activating substances are liberated.

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NO DISCUSSION

MUTATIVE BACTERIAL FERMENTATION

By *Martin Kristensen.*

As the articles which I have published on this subject in *Acta pathologica* (Vol. 17, p. 193; Vol. 19, p. 537; Vol. 20, pp. 530 and 791; Vol. 21, pp. 214 and 957) must make rather difficult reading for those who have not worked on the same problem, I shall roughly outline some of the principal features in the already published investigations and add certain more recent observations.

Mutative fermentation was first described by *Neisser & Massini* in 1906—07. What happens is that in a bacterial culture originally lacking the ability to ferment a sugar we suddenly find an individual possessing this power and thereby able to multiply at a higher rate than the rest of the culture. In plate cultures the mutation is recognized by the appearance of »acid knobs«, the acid of course being detected by a change in the colour of the indicator; as the indicator I have exclusively used bromthymol-blue, which is blue in the case of alkaline reaction, yellow in acid reaction. In fluid media there is a rather sudden production of acid after a few days, and when spreads are made from it on plates with the same sugar and bromthymol-blue (if seeding proceeds at a suitable point of time) we get a mixture of blue and yellow colonies.

To the casual observer it would seem that the mutation is the result of contact with the sugar; but after further consideration the question must be asked whether the mutation might not be spontaneous, so that the role of the specific medium consisted merely in promoting the growth of these spontaneous mutants and thereby making it possible to recognize them. And indeed, this view was advanced years ago by various authors, but at the time when I began to study it, only very little experimental material was available for appraising the question of spontaneous or induced mutation. For this reason I began to study the matter statistically.

My main experiment consisted of the seeding of about a thousand arabinose tubes with a strain of *Salmonella dublin*, whose propensity

for mutating with this sugar is well known, especially from a Danish work by *M. Christiansen*. Actually, these thousand tubes were not seeded at once, they being the aggregate of the tubes in a series of 96 tubes subcultured several times; all the tubes were observed until fermentation appeared. That the treating of the whole material together is justifiable is shown by the fact that the daily transplantations did not cause any real change in the disposition of the cultures to mutate under continued incubation. — Naturally, it is not enough to record the number of mutated tubes in proportion to the total number of tubes; from there we must try to get at the point that really matters: the mutation frequency in relation to the number of *bacteria*.

It became clear now in the first place that nothing could be established from the experiment as to the existence of a latent period in which no mutations occur at all. Still, the frequency of mutation was relatively low in the first few days. It increased gradually, and from about the third week after seeding till the termination of the experiment it remained fairly constant at about fifty times higher than the lowest value in the first few days.

This mutation may be compared with the transformation of a radio-active substance. If we take it that every change in the colour in a tube is the result of one mutation, and that each mutation leads to a visible change, we find that the lowest observed mutation frequency corresponds to a half value period of about 100 million years, the highest mutation frequency to a half value period of about two million years. Naturally, this result presupposes that the *progeny* of the mutating individuals are not included, and of course the non-mutating bacterium-individuals cannot exist for millions of years; nevertheless, the calculation provides a justifiable picture of the extreme rarity of the mutation process in the very cases where it is easily observed macroscopically; were it only a thousand times more frequent, it would not be recognized as a mutation at all; the fermentation would be considered to be primary.

In its early stages the mutation results in an intensely fermenting form, whereas in the later stages we find first a relatively weakly fermenting form, which, however, can be induced to continue mutating at one or more tempi into an intensively fermenting form.

We may now inquire whether this increase in the mutation frequency is due simply to the culture becoming older, or whether it is also due to the protracted contact with the arabinose. A separate test, in which the arabinose was not added until after cultivation had proceeded for some time, showed that both factors play a part.

Next, I experimented with the mutative fermentations of the typhoid bacillus.

In elementary bacteriological diagnostics we distinguish between Type 1, which ferments xylose promptly, and the less common Type 2, which does not ferment xylose in 24 hours, but may do so later

mutatively. Furthermore, strains of both Type 1 and Type 2 are capable of mutating with dulcitol, arabinose and rhamnose. In the first two cases mutation consists of the appearance of a fermenting, acid-producing form, whereas the mutation with rhamnose consists merely of the abolition of an initial growth inhibition. It now transpired that most Type 2 strains primarily are also strongly inhibited by xylose and yet they ferment this sugar, but very slowly. As in the rhamnose medium a mutation now proceeds, whereby the primary growth inhibition is abolished; but at the same time the low power of fermenting xylose disappears. It is only by a new mutation (which also seems capable of proceeding direct from the original form) that we get the form that is capable of a marked fermentation of xylose.

By mutating with xylose Type 2 is transformed into Type 1; it is generally possible, however, to distinguish this »artificial« Type 1 from the natural one by the circumstance that the former displays inhibited growth on media containing dulcitol. Here, however, it is quite possible for a new mutation to occur, whereby the growth inhibition is abolished, but at the same time the ability to ferment xylose is lost, so that we arrive back at the original form. This cycle can be reproduced several times.

Thus we have an example of two phenomena of mutation, a »positive« and a »negative«, which occur coupled; there are also examples of two coupled positive mutations, but as a general rule the various mutations occur independently. As we can distinguish three forms by the behaviour to xylose and two forms to dulcitol, arabinose and rhamnose, there is a priori a possibility of producing $3 \times 2 \times 2 \times 2 = 24$ different modifications of a given typhoid strain of Type 2 (including the original form). I have succeeded in producing them all.

I made various tests to elucidate the constancy of these forms. First, the fully modified form was subcultured a number of times on various media without loss of the acquired fermentation and growth properties. Next, all 24 forms were tested again after seven months had passed; by this time one of them had changed. Finally, all 24 forms were tested after $4\frac{1}{2}$ years; four of them had then altered in their behaviour to xylose and to dulcitol, whereas there was no instance of a change in relation to arabinose and rhamnose. (It may be possible to produce more stable forms).

Apart from *S. typhi* and *S. dublin*, there are several types within the *Salmonella* group which are slow to ferment various sugars. I have examined the more important of these and found that in some cases a primarily slow fermentation is involved, in others a mutative fermentation.

I have also studied a number of »coli-like« bacteria which ferment lactose or saccharose slowly. The result was a variegated picture of primarily slow fermentation, mutative fermentation and various com-

connection between the transition from S to R form and the fermentative change? Does the latter quality arise suddenly or is also this brought about through a gradual transition?

As far as I understood Dr. Kristensen had been able to make xylose-negative, dulcitol-positive strains become xylose-positive and dulcitol-negative by growing them in xylose medium. But when then these strains were transferred to dulcitol-medium, they again became xylose-negative and dulcitol-positive. These changes, I think, may hardly be designated as mutations in the de Vries sense of the term, which rather implies a sudden appearance of new permanent properties. In the above mentioned cases I find it more reasonable to look upon the new fermentative properties, which again may be lost, as an adaptation to the media.

M. KRISTENSEN: In epidemiological investigations the classification of typhoid bacilli in »type 1« and »type 2« has proved very reliable, as in the State Serum Institute we never have seen any sign of transformation of one type to another in a given patient or on transmission of the bacterium from one patient to another. The types produced artificially in the laboratory, indeed, are of no direct epidemiological significance.

In the old cultures, in which the lactose-fermenting *Salmonella* strain occurs, the rough form is often strongly represented. The fermentative mutants are sometimes markedly rough, but often relatively smooth, and as a rule they are easy to verify serologically.

As far as can be decided with the technique employed, the acquired capacity for lactose fermentation in the *Salmonella* strains is of mutation-like nature, as it is possible from the cultures in which the fermentation takes place to isolate both fermenting and non-fermenting forms.

ACQUIRED PENICILLIN RESISTANCE IN PATHOGENIC COCCI

By *Knud Rieiwerts Eriksen.*

In view of previous experiences with other antibacterial substances, it was only natural after the introduction of penicillin in the therapy at once to look into the question about penicillin resistance. Indeed a great number of works concerning this problem have been published already and it has been practicable in all the ordinary pathogenic cocci to produce an increased penicillin resistance by growing these microorganisms on penicillin-containing media.

The views concerning the resistance thus acquired have been widely divergent, as in many cases this resistance has been characterized as unstable, and the resistant strains have been designated as avirulent. On the other hand, the clinical literature has brought a good many examples of increase in the resistance of the cocci under treatment with penicillin.

The studies to be reported in the following were carried out with pneumococci, streptococci and staphylococci. In all the strains examined it was found practicable to produce an increase in their resistance after growing them for some length of time on fluid media with increasing concentration of penicillin.

After daily transfer of 10 pneumococcus strains for 2 months, their resistance was increased up to 60 times. In keeping with the findings reported by other investigators, all resistant strains showed a slower growth than the corresponding normal strains, weaker hemolysis on blood agar and smaller size of the individual cells, together with a few larger and irregular bacteria, often growing in chains. These strains have been under continuous control examination by means of the capsular swelling reaction, and the type character has remained quite unchanged.

In contrast to the findings of some other authors, it was possible in all cases — as a rule after very few mouse passages — to increase the virulence of the strains, which naturally was lowered consider-

ably after daily transfers in vitro through such a long period, so that they became almost just as virulent as the original strains. Only in one case — a pneumococcus Type 8, the resistance of which was increased about 20 times — was the virulence apparently lost completely. Serum broth cultures of this strain showed a large sediment with formation of long chains of large irregular bacteria; and on blood agar this strain produced no hemolysis. On mouse passages for 2 months, however, its virulence increased gradually, and at the same time its growth became normal again, while its penicillin resistance decreased to about one-half. In a few other cases the resistance decreased but very slightly through mouse passages. On the whole, however, the acquired penicillin resistance of these strains may be characterized as stable — even after mouse passages through a long period with cultures made on penicillin-free media.

With several of the resistant strains therapeutic experiments were carried out on mice with subcutaneous injection of a single dose of penicillin immediately after intraperitoneal injection of the respective pneumococci. Thus it was possible in vivo too to demonstrate an increase in the penicillin resistance of these strains, but the degree of resistance was always lower in vivo than in vitro. No doubt, this is due to the fact that infection with a resistant pneumococcus strain develops more slowly than does infection with the corresponding sensitive strain — in analogy with its lower rate of growth in vitro.

In keeping with the findings reported by other investigators, the sensitiveness of the strains in vitro to sulfathiazole remains quite unchanged on increase in the penicillin resistance. In therapeutic experiments, however, we meet with a rather considerable difference in this respect: successful treatment of the »resistant« infection requires considerably smaller doses of sulfathiazole than does infection with the corresponding penicillin-sensitive strain. Undoubtedly the explanation of this is also to be found in the slower development of the »resistant« infection.

In 6 strains of hemolytic streptococci, after daily transfer on penicillin-containing serum broth for about one month, the resistance was increased 100—400 times. On blood agar the resistant strains grew but very slowly and without hemolysis. The virulence of these strains was lost completely: even intraperitoneal injection of 2 cc. resistant culture was not lethal to mice. When the resistance was increased less than 10 times, the virulence was decreased but slightly. As soon as the resistance was increased 20—40 times, however, the virulence was very low, even though the capacity of the strain for hemolysis was not yet lost. Like the pneumococci, all the resistant streptococcus strains kept their penicillin resistance unchanged even on daily transfer on penicillin-free media through a long period.

In addition, 12 strains of staphylococcus aureus were tested in a similar way. After a few transfers on penicillin-containing broth the

resistance was already increased; and this was accompanied by changes in the morphology of their colonies: there was a decrease in the pigment production, and quite small non-pigmented colonies appeared. The number of these small variants of colonies increased with increasing penicillin resistance; and finally two strains showed these variants in pure culture. After daily transfers for 4 weeks the resistance was increased about 500 times. On determination of the resistance after the agar cup method as modified by *K. A. Jensen*, most of the strains showed only an insignificant bacteriostatic effect from 100 O. U. of penicillin per cc.

A few staphylococcus strains — those which showed the lowest percentage of small colony variants — presented some particular features on determination of the resistance after the agar cup method: perfectly clear zones of inhibition for as long as 10—12 hours — even with rather low penicillin concentrations — and then the inhibition zones were covered by a dense growth; but this growth could still be inhibited by the stronger penicillin concentrations. From these experiments it is evident that a »penicillin-resistant« staphylococcus culture consists of a mixture of rapidly growing penicillin-sensitive bacteria and slow-growing penicillin-resistant bacteria.

In contrast to the pneumococci and streptococci, the penicillin resistance of the staphylococci is soon lost on daily transfers on penicillin-free medium; and the above-mentioned small colony variants disappear at the same time. This is due in part to the fact that the small colony variants, which indeed represent the resistant fraction, are unstable and dissociate normal bacteria, and also to the circumstance that the normal, rapidly growing, penicillin-sensitive bacteria overgrow the resistant bacteria.

Later, with the same 12 strains of staphylococcus aureus, an increase in the resistance amounting to about 10,000 times was obtained. After this, most of the strains showed small colony variants in pure culture. After daily transfer on penicillin-free broth for 2 months all the strains still yielded pure cultures of non-pigmented colonies even though these now were a little larger than the original small colony variants. As early as after 2 weeks of daily transfer on penicillin-free broth the resistance had decreased quite considerably — to about 10 times the normal — and it kept at this level throughout the rest of the experiment. So the above explanation of the unstable resistance does not apply to all cases, as evidently the small colony variants may loose in resistance without dissociation of colonies of normal form.

The question about the underlying mechanism in the development of acquired resistance has lately been discussed a good deal. Previously it was the prevailing view that this was a matter of adaptation, through which the individual bacteria gradually got accustomed to grow on media with higher penicillin concentrations. Various authors have

claimed, however, that the resistance develops through mutation that arises independently of the presence of penicillin.

Of course, the formation of the small colony variants indicates plainly that mutation takes place, but it does not tell us anything as to whether this mutation is independent of the penicillin. Small colony variants are also seen now and then in older cultures, but I have not been able in such cases to demonstrate any increased resistance. That this theory might be correct, however, is suggested by the circumstance that strains which otherwise readily develop a marked degree of resistance may now and then be very difficult or impossible to render resistant. This was seen, for instance, in the case of one strain which with the agar cup method now and then was found not to develop the opaque zone of relative inhibition that — according to *Vesterdal* — probably appears through mutation.

The question whether acquired penicillin resistance is a phenomenon of clinical importance has been estimated very differently. From the results of their experimental investigations, most authors have been inclined to look upon a possible development of resistance under penicillin treatment as being of no significance whatever, partly because the development of such resistance always was found to be accompanied by a loss of virulence, partly because the resistance in some cases is unstable — something that has been reported to apply not only to staphylococci but also to streptococci. My own studies have shown that it is quite practicable with pneumococci to obtain a fairly great increase in the penicillin resistance without their virulence being lost, and thus we cannot refute the possibility of a development of resistance under penicillin treatment. But, as far as I know, this possibility has never been seen to materialize — perhaps, in view of previous experiences with the sulfonamide therapy — because the doses of penicillin employed have always been large.

Several reports have been published, however, on the development of penicillin resistance under treatment of staphylococcal infections, in particular osteomyelitis. But the penicillin-resistant strains appearing under such treatment are found to produce penicillinase — something that never is seen in strains that are rendered resistant in vitro. Now, as is well known, some staphylococcus strains are found to be primarily resistant just because they form penicillinase. So to me it seems not improbable that in the cases where a resistance has been described to develop under the treatment of staphylococcal infection this may have been due either to a secondary infection with such primarily resistant strains or to an initial mixed infection. Indeed, the appearance of penicillin-resistant bacteria during the treatment is no uncommon phenomenon, as secondary infection — for instance with *Ps. pyocyaneus* or *E. coli* — is often encountered.

So, I think, the greatest danger lies in the possibility of a gradually increasing tendency of primarily resistant strains — just as is seen, for instance, in the sulfonamide treatment of gonorrhea.

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DISCUSSION.

L. O. BORGES: I have been very interested in hearing Dr. Eriksen's paper, and I wish to make use of this occasion to seek an answer to a question which the clinicians often ask us laboratory workers when we find and report an otherwise penicillin-sensitive microbe as penicillin-resistant.

The question is: May it be of any use to employ penicillin in such a case?

Here I have in mind our experiences from the Ullevaal Hospital as far as staphylococci are concerned. A material from our laboratory contained about 10—12 % primarily resistant strains of *Staph. aureus* showing a variable, though always high, degree of resistance, i. e., from 800 to 500 penicillin units per ml. broth.

What advice are we to give the clinicians in such cases?

Even though these strains produce penicillinase in vitro, may it not prove profitable anyhow to try penicillin therapy?

O. SIEVERS: In a case of acute osteomyelitis, *Staph. aureus* was obtained in pure culture. From the primary blood plate, showing only one kind of *staph. aureus* colonies, 30 colonies were picked out for further cultivations. Each of the resulting pure cultures was tested separately with penicillin. In 10 % the bacteria (3 colonies) were quite resistant, while the remaining showed sensitiveness as the control strain obtained from Fleming. After treatment of the patient with penicillin, a repeated examination showed exclusively resistant strains of *Staph. aureus*.

K. R. ERIKSEN: As is well known the penicillin-resistant staphylococci are resistant only on behalf of their production of penicillinase; in reality they are just as sensitive as ordinary sensitive strains. Just the same phenomenon is known for the anthrax bacillus and infections with anthrax have successfully been treated with penicillin. Therefore, I suppose that the failure of penicillin therapy often observed in osteomyelitis may not be due to the »penicillin resistance« of the infecting organism but to some other factor. The occurrence of persisters presumably is not a significant factor as persisters in vitro are gradually lysed by penicillin. Perhaps the most important factor is the impermeability for penicillin of the affected tissues in osteomyelitic processes.

O. SIEVERS: In his mention of severe cases of osteomyelitis, Dr. Eriksen calls attention to the hindrance to the penicillin effect which the tissues may effectuate through their impermeability. In this

connection, I think, it will be appropriate to mention a case of osteomyelitis treated by Dr. Levin in Gothenburg, in which the penicillin therapy was combined with dicoumarin (AP). So far, this treatment appears to have given a favorable result. The staphylococci in question were not affected in vitro by penicillin or dicoumarin (AP), nor by a mixture of the two remedies.

THE BACTERIOLOGICAL ORIGIN OF A SPREADING FACTOR PRESENT IN THE NASAL SECRETION*)

By S. Bergqvist and Th. Packalén.

In 1928, Duran-Reynals demonstrated that the spreading of infectious agents in the skin is considerably increased if they are injected together with testicular extract (5, 6). Later he showed that extracts of many other organs had a similar spreading effect. Moreover, it was found that such spreading factors are also produced by certain bacteria (staphylococci, streptococci and pneumococci, *Cl. welchii*, etc.), and occur in the venom of snakes and insects. For literature on this subject, the writers refer to Duran-Reynals' monograph in the *Bact. Rev.* 1942 (7).

The spreading factors are, as a rule, enzymes -- hyaluronidases -- which hydrolyse hyaluronic acid. This latter substrate is present, *inter alia*, in the ground substance of the connective tissue, and in the capsules of certain bacteria, *e. g.* streptococci (11). It is present in particularly large quantities in the vitreous humour and the umbilical cord, and is usually prepared from the latter. The acid, which forms highly viscous solutions, belongs to the group of mucopolysaccharides, which have been thoroughly studied in Sweden by Blix and co-workers (3, 9).

The hyaluronidase activity can be measured by viscosimetry, or determination of the amount of glycuronic acid and N-acetyl glucosamine formed by the hydrolysis of the hyaluronic acid, or by the so-called mucin-clot prevention test (M. C. P.), in which the capacity of the mucopolysaccharide for forming a typical "mucin clot" on the addition of acetic acid is titrated.

In the years immediately after Duran-Reynals' discovery, numerous investigations concerning the occurrence of spreading factors in nature were made. Hanger observed in 1931 (8) a spreading effect in a great number of nasal washings from persons with acute coryza, but not infrequently in washings from healthy persons, too. He associated

*) Aided by grants from the Swedish National Association against Tuberculosis.

the presence of the spreading factor on the nasal mucosa with a filtrable agent isolated by Dochez and co-workers in 1931 (4) which, inoculated into human beings, caused coryza. He was, however, unable to demonstrate any spreading effect in tissue cultures harbouring this agent. Hanger did not enter into further discussions concerning the origin of the spreading factor in the nasal mucosa, and no investigation of this problem appears to have been carried out later.

During the work which has been carried out at St. Göran's Hospital, at the instigation of Westergren, in order to study the significance of multiple infections in tuberculosis, attention has also been paid to the spreading factors (2, 12). Hanger's observation caused an investigation to be made to determine, whether the spreading factors in the nasal washings could be related to the occurrence of hyaluronidase-producing bacteria in the nose.

Methods: Swabs were taken from the nose of a number of persons, and at the same time nasal washings were performed with approximately 15 ml. sterile broth. Eighteen-hours cultures of the coagulase-positive staphylococci isolated from the nose were examined with the mucin-clot prevention test for hyaluronidase activity. The nasal washings were immediately Seitz-filtered, and concentrated by freeze drying to approximately one-fifth of the volume, and 0.8 ml. of the concentrate was injected, together with an indicator, into the shaved skin of a rabbit. The indicator consisted of a mixture of 0.1 ml. haemoglobin solution (washed sheep blood corpuscles, haemolysed by freezing and diluted to double the volume with physiological saline solution) and 0.1 ml. 4-days Dubos' culture of the H 37 strain of tubercle bacillus. Each rabbit got four washings in a row on one side, whilst on the other side four controls were placed at corresponding sites. These controls consisted of the indicators mixed with sterile plain broth, which had been concentrated to one-fifth of the volume. The areas of the skin coloured brown by the haemoglobin were measured after approximately 18 hours, and the areas of the developing tuberculous cutaneous lesions after a further two weeks. Both these readings were in complete agreement. The difference between the areas obtained by the nasal washings and the control broth respectively was expressed as a percentage of the latter area. The results are seen in Table 1.

In the table, the nasal washings are divided into a *Staphylococcus aureus* positive and negative group, respectively. It is seen that all nasal washings from staphylococcus carriers show a more or less pronounced spreading effect, when compared with the controls. A rough relationship between the degree of spreading and the amount of staphylococci (+++ to +) on the nasal mucosa seems to exist. In those cases where *Staphylococcus aureus* was not found, no spreading factor was, as a rule, seen in the nasal washings. In Case H 35 there was, however, a distinct spreading effect despite the fact that hyaluronidase-producing bacteria were not isolated. Bacteriological and serological studies carried out by Adamson (1) and by the present

Table 4.

Connexion between occurrence of hyaluronidase-producing staphylococci and presence of spreading factor in the nose.

Nasal washing	Staph. aureus	M. C. P. ¹	Spreading	Nasal washing	Staph. aureus	M. C. P.	Spreading
			per cent				per cent
H119	+++	1/100	+530	H135	—		+65
H117	+++	1/4	+233	H121	—		+14
H14	+++	1/4	+206	H134	—		+9
H115	+++	1/100	+88 to 218 ²	H114	—		+4
H123	+++	1/4	+190	H137	—		-9
H18	+++	1/10	+55 to 200 ²	H110	—		-13
H11	+++	1/4	+162	H112	—		-21
H122	+++	1/4	+112	H120	—		-22
H133	++	1/4	+102	H130	—		-24
H17	(+)	1/4	+87	H132	—		-32
H118	+++	1/1	+80				
H124	+++	1/10	+50				
H29	+	1/4	+49	H128	— ³	1/256	+160
H116	+++	1/4	+40				
H131	+	1/4	+28				
H111	(+)	1/4	+18				

¹ Mucin-clot prevention titre.

² Several experiments.

³ Hyaluronidase-producing pneumococci almost in pure culture.

writers (13) have shown that increases in the antistaphylolysin titre, even when clinical symptoms of infection are absent, are, as a rule, connected with a staphylococcal infection in the upper respiratory tract. From there the staphylococci easily spread to the cervical and mediastinal lymph glands. In the patient in question the antistaphylolysin titre increased definitely during the observation period, thus indicating the presence of a staphylococcal infection somewhere within the organism. It is not improbable that staphylococci, although not found in the cultures, were nevertheless present in the nose or sinuses. This would explain the presence of hyaluronidase in the nasal secretion.

In another case, H 28, there was also a considerable spreading effect, although no staphylococci were seen in the culture. Here, however, the cause of the spreading effect was apparently the presence, almost in pure culture, of pneumococci which were very strong hyaluronidase-producers. The patient was suffering from coryza.

However, other cases which had coryza, but no staphylococci or pneumococci on the nasal mucosa, were without any spreading effect whatsoever.

Repeated tests made on a few persons revealed that, as soon as the

Staphylococcus aureus disappeared from the nose, the spreading effect of the nasal washings was also lost. Indeed, the connexion between the spreading factor in the nasal secretion and the hyaluronidase-producing bacteria in the nose seemed to be a very close one.

The results of investigations published recently by Hechter (10) indicate that the spreading factor has no effect, when applied externally to the skin or mucosa, in other words, as long as no increase of interstitial pressure in the tissues is brought about, as is the case when a fluid is injected. It can thus scarcely have any significance for the spreading of bacterial infections along the mucosa of the respiratory tract. If, however, the hyaluronidase-producing bacteria penetrate into the tissues and give rise to inflammatory oedema with increased interstitial pressure in the tissues as a result, the enzyme in all probability, will play a rôle in the further spread of the infection. It is likely that the spreading factor then supports the invasion of such concomitant bacteria, too, which do not themselves produce hyaluronidases. Certain investigations have already been carried out at St. Göran's Hospital, in order to study in animals the possible influence of the spreading factor on tuberculous infection. Observations made hitherto appear to indicate that an experimental infection with tubercle bacilli, together with hyaluronidase-producing bacteria, results in a more rapidly progressing tuberculosis than such an infection together with bacteria not possessing this property.

Summary.

The presence of spreading factors in the nasal secretion is intimately connected with the occurrence of hyaluronidase-producing bacteria on the nasal mucosa. As a rule, these are coagulase-positive pathogenic staphylococci, exceptionally pneumococci. The significance of a more or less permanent existence of bacterial spreading factors in the upper respiratory tract is discussed in relation to the invasion and spreading of other infectious agents.

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NO DISCUSSION

NON-SPECIFIC ANTISTREPTOLYSIN REACTIONS AND SERUM (OR PLEURAL-EXUDATE) CHOLESTEROL

By Thorolf Packalén.

Strong increases in antistreptolysin titre, AST, independent of specific stimuli occur (1) in sera (and pleural exudates) contaminated with certain bacteria, or (2) treated with acid or alkali, (3) in most hepatitis sera, and (4) in pleural exudates of some duration.

The non-specific nature of these rises was confirmed by the non-absorbability of the inhibitory factor with specific streptolysin, and the blocking of the inhibition by precursory adsorption of streptolysin to the red cells at 0° C.

In electrophoretic separation experiments the inhibitory factor was found to remain chiefly in the β - and α -globulin fractions, i. e., just those fractions in which the serum cholesterol accumulates, whereas the antibodies are found in the γ -globulin fraction.

Lipid extraction of sera and exudates abolished non-specific AST reactions in them, and rendered them simultaneously unable to respond with a rise in AST to bacterial contamination or treatment with acid or alkali. Specific antistreptolysin, on the other hand, remained unaffected by this procedure.

It has not been possible to prove, by cholesterol analyses, that an increase of "free", i. e. not esterified cholesterol — as seen in hepatitis sera — should be considered the consistent explanation of most non-specific rises in AST. However, the hydrolysis of ester cholesterol may play some part in the origination.

It is suggested that the primary mechanism of the non-specific AST reactions is a breaking up of the lipoprotein complexes, in which the serum lipids are supposed to occur. The uncovered active OH groups of the freed cholesterol combine with the streptolysin molecules, thus preventing them from attacking the red cells. The breaking-up process may be brought about, *inter alia*, by the action of acid or alkali, or bacterial enzymes, or enzymes liberated or activated

through the disintegration of liver cells in hepatitis, or leukocytes present in pleural exudates of some duration.

Attention is called to the possibility of using the AST reaction as a sensitive biological indicator for free cholesterol.

A detailed report will be published elsewhere.

DISCUSSION

O. SIEVERS: Anybody who has performed an AST determination knows that icteric sera often are not readable. In order to throw some light on this question it will be appropriate here to give the outcome of an examination of 108 sera from patients with some liver lesion. Twelve of these sera showed normal values, AST could be read as usual, and the number of units per ml. was not too high. Whether the patients recently — or at the taking of the blood sample — possibly may have had a streptococcus infection is not known; nor is this of any importance when it comes to answer the question whether there be any parallelism between the difficulty in reading AST (or abnormally high values) and the results obtained with the following chemical reactions: Meulengracht, serum phosphatase, serum citric acid, and thymol flocculation. In the 96 cases where some of the performed reactions gave increased values, the AST values varied greatly. Besides entirely normal values, several cases showed so strong reactions (8000—> 30,000 units/ml.) that the results could not be accepted even though they were obtained again on repeated examination. In other cases the results were unreadable because no definite limit could be established for the hemolysis.

No correlation was found between high AST values and high values for serum citric acid or serum phosphatase. In contrast hereto, high values for Meulengracht and for thymol flocculation were often found together with abnormally high or unreadable AST values. Sera with high Meulengracht values might sometimes give acceptable values for AST, but — with two exceptions — only provided that the thymol flocculation at the same time gave normal values. On the other hand, a high value for thymol flocculation is not enough to interfere with AST — which requires that the serum at the same time contains bile pigment, Meulengracht preferably 30 or higher. The above-mentioned exceptions show, however, that in the serum there is bound to be some other, hitherto unknown, factor that may influence AST. Of course, the chemical examinations mentioned are not to be performed on every serum. But, if AST gives too high values, the causes here discussed should be looked into.

WESTERGREN: I wish to compliment Mr. Packalén on his brilliant and systematic investigations, which perhaps may prove to be of

more comprehensive interest than merely as a theoretical explanation of a serological phenomenon. But chiefly I have asked permission to speak on account of some remarks made by Mr. Sievers about the clinical aspects of a non-specific high AST in cases of jaundice.

I have long been interested in the problem of the liver function, and I have tried to find some connection between these questions. Concerning the difficult liver functional tests, only the Takata reaction appears to have some relation to AST. Icteric sera need not necessarily give a non-specific increased AST, even though exceptions are uncommon. Even more rare is a non-specific increased AST in disease of the liver without icterus — the so-called *hepatitis sine ictero* — of which I have observed at least one case. Further, as just mentioned by Mr. Kalbak, it may occur in nephrosis, but I wish to emphasize that there are cases of nephrosis without any such increase in AST.

In addition, it should be pointed out that this phenomenon may be of interest also in pleurisy. But the non-specific increased AST in pleuritic exudates probably is still more closely related to the age of the pleurisy than was evident from the picture just shown by Mr. Packalén. In particular, it is to be emphasized that the exudate in entirely acute pleurisy appears not to show this reaction, which seems to appear after about one month. In cases showing such a non-specific increase in AST at an earlier stage, evidence can very often be obtained to the effect that the pleurisy must be assumed to be older than it first looked clinically.

To me it seems rather likely that in pleurisy as well as in diseases of the liver this non-specific rise in AST may to some degree be connected with the prognosis of the disease — especially in that a low increase, or no rise at all, is found exclusively in cases taking a favourable course.

As to an explanation of the phenomenon, I wish to emphasize the relation to cholesterol pointed out by Mr. Packalén, but also that there is no direct relation to any now definable form of cholesterol.

O. SIEVERS: In reply to Dr. Westergren's remark about »unspecific« AST values being obtained together with a positive Takata reaction, I wish to point out that the outcome of the Takata reaction is not recorded in my tables because this reaction was not performed on all the sera. However, »unspecific« AST values have been obtained with Takata-positive as well as Takata-negative sera.

KALBAK: Studies on the unspecific reactions are of importance, as presumably through such studies it may be possible to clear up certain aspects of the very nature of hemolysis itself. I hold the same view as has been advocated by Todd & Hewitt and by Packalén: that the cholesterol content of the serum probably plays a certain rôle

in the so-called unspecific reactions. Serum from patients with high serum cholesterol (*e. g.*, parenchymatous liver lesions, genuine nephrosis, and other diseases with lipemia) (for instance, Simmonds' disease) gives such characteristic reactions, which differ quite distinctly from »normal« reactions by having a low hemolysis curve — in contrast to the normal reactions, for which the hemolysis curve is rather abrupt. Thus, in practice, distinction can be made at once between an abnormal reaction with high »unspecific« AST and a true increased AST. So the clinicians need not be afraid of any mistake on the part of the laboratory. We may reckon, I think, that about $\frac{1}{4}$ % of all reactions turn out to be such abnormal reactions, and in the »State Serum Institute« we designate them as »non-readable«.

Presumably such a reaction involves an inhibition of hemolysis, and apparently the cholesterol content plays a certain rôle in its production, but the mechanism of it is still obscure. As already pointed out, it is highly important to get this mechanism cleared up, for then presumably we shall attain a better understanding of the nature of the reaction itself.

T. PACKALÉN: Dr. Sievers made a comparison between the anti-streptolysin titers in hepatitis sera and the outcome of certain functional liver tests, including Meulengracht's reaction, on which account I find it appropriate to present an additional table. From this it is evident, while the increased titers in some hepatitis sera appear to be of entirely non-specific nature (no absorption with active streptolysin; total blocking of the inhibition by precursory adsorption of streptolysin to the red blood cells at 0° C) the increased titers in other cases appear in part to be elicited by specific antibodies (the inhibition is reduced in part after specific absorption; it cannot be eliminated completely by preceding adsorption to the red blood cells). The existence of a specific antistreptolysin component in these sera is further confirmed by the observation that in these cases the titers were found to have been increased before or after the hepatitis. The irregularities in the correlation between the rise in AST and the increase in the Meulengracht values which Dr. Sievers had in his diagram, and which Dr. Westergren also had observed a few times may be explained by the occurrence of such combined specific and non-specific increases in titer. On the other hand I am fully convinced that the non-specificity of hepatitis sera is not induced directly by the changes in the serum that give rise to the increased Meulengracht values. The latter changes run parallel — though exceptions do occur — with the breakdown of the lipoprotein complex which, in my opinion, is the underlying cause of the increase in AST in hepatitis serum.

I fully agree with Dr. Kalbak that the non-specific AST reactions described and studied by me do not disturb the routine AST determinations in any degree worth mentioning. But we have to keep in mind

that no particular diagnostic significance may be assigned to increased AST values in hepatitis sera if we fail by the aid of special examinations to decide when they are specific, when non-specific.

As already pointed out by Dr. Westergren, a high AST in a presumably fresh pleural exudate is reason enough to make further inquiry as to the age of the pleurisy.

As to the question raised by Dr. Löfgren — why only certain bacteria give rise to non-specific AST in sera — I am not able yet to give an adequate answer based on actual experimental studies. It looks, however — as already touched upon by Todd — as if it were primarily a matter of bacteria capable of producing plenty of proteolytic enzymes when such non-specific increases in AST occur. The enzymatic breakdown of the protein component should then bring about the decomposition of the lipoprotein complex which I assume to give rise to the increase in AST.

Finally, as to the question whether the rise in AST produced by bacteria might not have something to do with their respiratory ferments, I merely wish to mention that I too have considered this possibility. But, for various reasons, it has been refuted. The absence of any increase in titer when the sera before infection are lipid-extracted would be difficult to explain if the cause of the increased titer was to be looked for in an inactivation of the streptolysin resulting from oxydising processes of the bacteria, or in changes in the redox potential produced by them. Besides the magnitude of the latter is all too slight for the production of such increases in AST as we are dealing with here.

ON THE OCCURRENCE OF ANTISTREPTOCOCCAL O AGGLUTININS IN THE SERUM OF PATIENTS WITH ACUTE OR CHRONIC POLYARTHRITIS

By *Karl Emil Thulin.*

Attempts have been made to demonstrate specific antibodies against streptococcal antigens in various diseases.

Thus Cecil, Nicholls and Stainsby in 1931 observed that sera from patients suffering from chronic polyarthritis agglutinated streptococci. Later Dawson, Olmstead and Boots were able in extensive studies to verify these results and show that it was the beta-haemolytic streptococci that were agglutinated by these sera.

They used living bacteria belonging to the A group, incubated for a short time. In 1936 Dawson found positive reactions in 51 % of cases of chronic polyarthritis.

Using a slight modification of Dawson's technique, Kalbak in 1946 demonstrated positive agglutination reactions with living human-pathogenic streptococci in 76 % of patients suffering from chronic polyarthritis but in only 10 % of cases of rheumatic fever. The control material showed only 1.5 % positive agglutination reactions.

These streptococcal agglutinations, demonstrated by Dawson et al. and, lately, by Kalbak are likely caused by an antibody against a surface antigen, which chiefly appears in chronic cases, and the nature of which has not been established.

The chief difficulty in these reactions is to obtain antigens that do not show spontaneous agglutination.

By using precipitation tests, Rebecca Lancefield overcame the difficulties arising from spontaneous agglutination and in this way she laid a solid serological foundation for classification of the bacteria belonging to the streptococcus group.

But the antigens obtained in this way cannot be employed for agglutination tests, and obviously agglutination reactions are simpler reactions than precipitation reactions.

The experiments on which this paper is based, therefore, were carried out with a view to this question: Is it possible in principle to apply

the same antigenic structure to the bacteria of the streptococcus group as has been worked out by Kauffman-White for the *Salmonella* group?

From this starting point experiments have been carried out to establish an antigenic schema in which, especially, the conditions in the serology of the colon group have served as guiding principles (cf. Vahlne 1945).

As time does not permit any thorough review of this antigenic schema, only the various antigenic components will here be outlined briefly as a basis for the following account.

Nor will any detailed comparison be made between the findings to be reported here and the Lancefield antigenic schema.

For the sake of illustration, the present antigenic structural schema may conveniently be presented in this way.

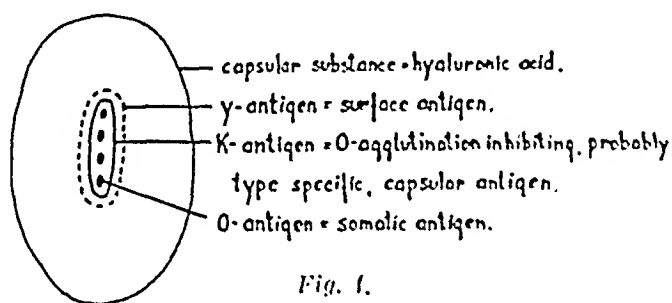


Fig. 1.

Y antigen: Kalbak's antigen for agglutinative reactions in chronic polyarthritis.

K antigen: O agglutination-inhibiting, probably type specific, antigen occurring in virulent streptococcus strains pathogenic to man.

O antigen or somatic antigen.

Capsular substance = hyaluronic acid.

These antigens are obtained as follows:

Y antigen, through cultivation in peptone broth ad modum Dawson-Kalbak.

K antigen, through decomposition of the capsule with hyaluronidase prepared from testicular extract.

O antigen, through growing the strain in placenta broth for 16 hours, then autoclaving for 2 hours at 120°.

Capsular substance, with problematic antigenic character, in the form of well-developed capsules after one or more mouse passages, until mucoid growth appears on 5 % ascites or 10 % horse serum blood agar plate after incubation at 37° for 12 hours.

Antigens thus obtained show no spontaneous agglutination. In the following an account will be given of the experiments with the O antigen.

On autoclaving of the bacterial cultures at 120° for 2 hours — after the method employed by Vahlne in his serological studies on the colon group — a streptococcal antigen is obtained that has no tendency to spontaneous agglutination. Immunization of rabbits with this antigen gives specific sera with titers from 1:640 to 1:5120.

The O antigens thus obtained are partly identical with Lancefields group substance antigen. By means of O absorptions after Castellani, these O antigens can be shown to be composed of different partial antigens. Thus, within the human-pathogenic group A streptococcus strains may be demonstrated with various combinations of partial antigens (the partial antigens are recorded in Roman figures) as for instance:

$$A\ 1 = I, V, XI; \quad A = I.....?; \quad St.\ 31 = III, V,$$

where I is common to strains belonging to group A, V to group C, III common to certain strains of group D, and XI to certain strains of group L.

The question then arises: Is it possible to demonstrate antibodies aimed against these O antigens in patients with streptococcal lesions?

Are such agglutinins encountered also in other diseases, especially those which long have been looked upon as associated with streptococcal infection, *e. g.*, acute and chronic polyarthritides and nephritis? In order to elucidate these questions, a number of experiments have been carried out with the following technique.

Preparation of antigen:

After incubation of the placenta broth culture (Vahlne 1945), centrifuging and washing in phosphate buffer with pH 7.8. Then autoclaving at 120° for 2 hours. The autoclaved antigen is washed twice with the buffer and then diluted to a density corresponding to $1\ E = 10^9$ bacteria per ml. The antigen is stored in refrigerator at +4° for up to 3 days, after which fresh antigen is prepared.

Agglutination technique:

Widal tubes with a diameter of 9 mm are used.

Serum dilutions from 1:20 to 1:320 are made up, 0.2 ml of each dilution being placed in the respective tubes. Then 0.2 ml antigen is added to each tube, and — after gentle shaking — the tubes are incubated in water-bath at 52° for 2 hours. The tubes are left standing in refrigerator +4° over night, and the results are read the following day.

The highest serum dilution showing a positive agglutination with »clearing« of the mixture gives the agglutinative titer of the serum in question.

Two antigens belonging to group A have been employed.

Patient material.

The patient material here examined is divided into 4 groups:

Group I Control material.

Group II Acute streptococcal infections: Scarlatina.

Acute tonsillitis.

Group III Rheumatic fever.

Group IV Chronic polyarthritis.

Control material.

The control material falls in two groups:

One group comprises 158 Wasserman-negative sera from patients treated in the hospital for diseases other than polyarthritis and nephritis within the summer half-year of May—October. The other group comprises patients of the same category admitted to the hospital during the winter half-year of November—April.

A survey of the results is given in Table 1.

Titer	$<1/20$	$1/20$	$1/40$	$1/80$	$1/160$	P_{60}	N_{60}	Total No. of cases.
	Neg.			Pos.		%	%	
Summer half-year	138	15	3	1	1	3.2	96.8	158
Winter half-year	59	25	8	2	1	11.6	88.4	95

Table 1. Normal serum controls.

From Table 1 it will be noticed that, with the pathological titer limit of 1:40, positive reactions were obtained in 3.2 % of the summer group, whereas the winter group gave positive reactions in 11.6 % of the cases. This is interpreted as signifying an increased number of streptococcal infections in the winter half-year.

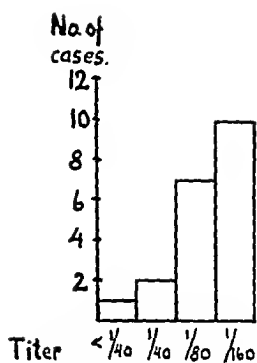
Group II.

In order to ascertain whether acute streptococcal infection may give rise to a demonstrable O agglutinin content of the serum, 20 scarlatinal patients and 20 patients with acute tonsillitis were examined 2—3 weeks after the onset of their illness.

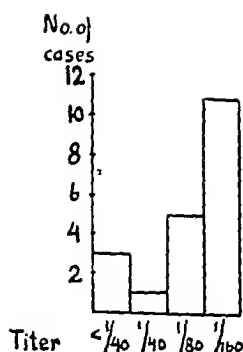
From Table 2 it is evident that 5 % of the scarlatinal patients and 15 % of the tonsillitis patients gave a negative reaction. The remaining patients gave a positive reaction at various titers, with a maximum titer value of 1:160.

Fig. 2 presents some agglutination curves for uncomplicated cases of acute tonsillitis.

Evidently the maximum titer value appears to be reached relatively rapidly in the second week of illness, whereafter it falls off somewhat slowly or, in exceptional cases, rather rapidly.



Scarlatina. 20 cases.
(2-3 weeks)



Acute tonsillitis. 20 cases.
(2-3 weeks)

Table 2.

Thus an agglutinating antibody is demonstrable also in acute cases of streptococcal infections.

A similar course we also find for another acutely appearing streptococcal antibody, the antistreptolysin or AST, which was first demonstrated by Todd in 1932. Many investigators in England, U. S. A. and Scandinavia have been able to verify that a rise in AST occurs in diseases caused by hemolytic streptococci after a certain latent period varying from 1 to 2 weeks.

How do AST and the O agglutinins stand mutually?

In order to elucidate this question, the AST and the O agglutination reaction in a number of cases of acute nephritis have been observed parallelly, but it is yet too early with certainty to say anything about their relations.

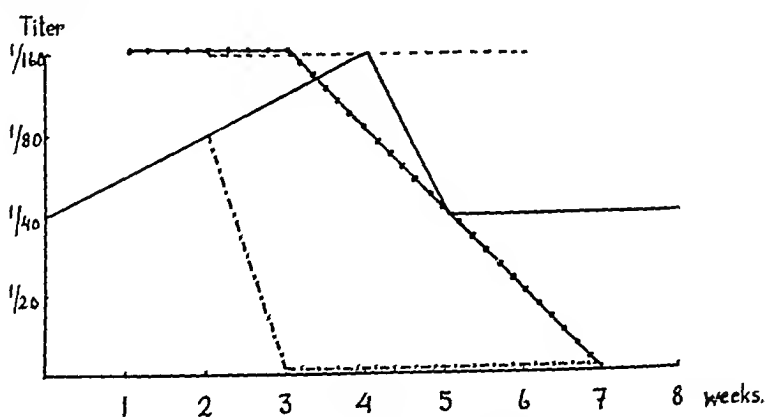


Fig. 2. Agglutination curves from uncomplicated cases of acute tonsillitis

There seems, however, to be a certain correspondence in the titer course with partly parallel shifts in the antibody titer.

The time does not permit a further analysis of this parallelism, but it is likely that the O agglutinins appear both in acute and chronic cases as distinguished from the AST, which as a rule only occurs in acute streptococcal diseases.

Group III.

This group comprises 28 patients who became ill with the clinical features of rheumatic fever. On an average, the agglutination tests were performed about 6 months after the onset; in 4 cases, however, after 3 months.

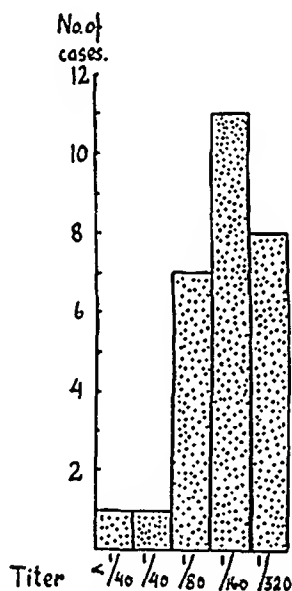


Table 3. Rheumatic fever. 28 cases.

Table 3 shows the highest titer values obtained with the two antigens employed. Negative reactions were found in 3.5 % of the cases.

Group IV.

This is the largest group, comprising 189 cases of chronic polyarthritis.

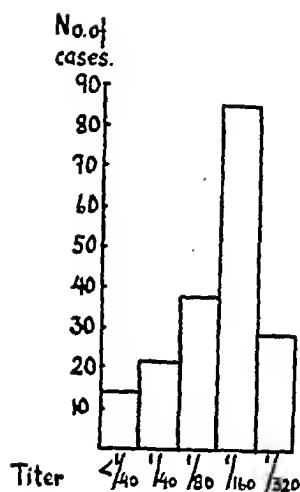


Table 4. Chronic polyarthritis. 189 cases.

This table gives a general survey of the results. It will be noticed that the tests were negative in 7.4 % of the cases, while in all the others they were positive, with titers varying from 1:40 to 1:320 — i. e. 92.6 % positive reactions. A titer value of 1:160 or higher was obtained in about 60 % of the cases.

Which are the relations between these agglutination reactions and the reactions with living antigens — by the author called Y antigen?

This may be revealed by absorption experiments with autoclaved antigens, i. e., O antigens with various combinations of partial antigens.

Antigen	Titer before absorption.	Titer after abs. O-ant.=I,V,XI	Titer after abs. O-ant.=IV.
y	160	160	160
O-ant.=I,V,XI	160	0	160
O-ant.=IV.	0	0	0

Table 5.

Agglutination reactions before and after absorption experiments with O-antigen=I,V,XI, and IV from one case of chronic polyarthritis.

This table shows agglutination reactions before and after absorption with two different strains, one from the A group with the partial antigens I, V, XI the other with the partial antigen IV, i. e., belonging to the Lancefield B group.

Before the absorption the Y antigen produced a titer of 1:160, the O antigens I, V, XI also 160, while IV did not show any reaction. After absorption with the O antigens I, V, XI, the Y antigen titer still was 160, while the O agglutinins were completely absorbed. Absorption with the O antigen IV, on the other hand, did neither change the reactions to the Y antigen nor the O antigen.

From this we may conclude that the reactions with living streptococcal antigen ad modum Dawson-Kalbak and the antoclayed antigen are of different character.

In cases of chronic polyarthrits there are two kinds of antibody against the human-pathogenic streptococci, namely the Y agglutinins and O agglutinins.

In analogy with the Vi agglutinins in *Salmonella* infections Y agglutinins occur in the chronic cases, whereas O agglutinins are demonstrable early in the infection and remain as long as the infection lasts.

Diagnosis	Aggl. Cases pos. in %	Aggl. Cases neg. in %	Total No. of cases
Scarlatina	19 = 95%	1 = 5%	20
Acute tonsillitis	17 = 85%	3 = 15%	20
Rheumatic fever	27 = 96%	1 = 3%	28
Chronic polyarthrits. . .	175 = 92%	14 = 7%	189
Nephritis, acute.	47 = 92%	4 = 8%	51
Nephritis, chronic.	59 = 95%	3 = 4%	62
Total:	344 = 93%	26 = 7%	370

Table 6.

Thus, 93 % of the patients in the present material gave positive reactions with various diseases in which streptococci play -- or are assumed to play -- a decisive role.

Thus, 93 % of the patients in the present material gave positive reactions. In contrast hereto, about 10 % of the subjects in the normal material gave positive reactions.

The present findings may be summarized as follows:

1. Through application of experiences from the serology of the *Salmonella* and colon groups, the Lancefield antigenic schema may be further elaborated by the advancement of the theory: that the human-pathogenic β -haemolytic streptococci possess a somatic O antigen and a capsular K antigen that inhibit O agglutination.
2. By means of these O antigens of streptococci belonging to the human-pathogenic group A it is possible to demonstrate the presence of antibodies aimed against this antigen -- O agglutinin -- in the blood of patients suffering from diseases in which streptococci play -- or are assumed to play -- a decisive role.

3. Like the AST, these O agglutinins occur in acute cases but, contrary to AST, also in chronic infections.
4. In cases of chronic polyarthritis two kinds of agglutinins aimed against different antigens in the human-pathogenic streptococci can be shown, the Y agglutinins in about 70 % and O agglutinins in about 90 %.

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DISCUSSION

H. KALBAK: Dr. Hedlund has tackled the task of elucidating the relation between the AST and the agglutinative titer. As far as I can see, he has arrived at the same result as I, namely: that the two reactions do not run parallel — as perhaps they might have been expected to do. Both reactions signify an infection with hemolytic streptococci, but at the same time either of them is an expression of its own differing mechanism of infection. AST illustrates the toxic function of the streptococcal infection, and it is due to the formation of antitoxic antibodies, whereas the agglutinative reaction merely signifies the presence of the streptococcal body (living or dead) in the organism, being thus entirely an antibacterial reaction. Therefore, the two reactions need not go together even though both are produced by the same bacteria.

In the acute phase of the streptococcal disease it is especially the antistreptolysin reaction that turns out positive. If the infection persists, spreading in the organism, and if the disease takes a chronic form, especially through concomitant joint symptoms, the agglutinative reaction gradually becomes positive, at first quite weak (agglutinative strength 1 in the first dilution) later it becomes stronger. On this point my experiences are in keeping with these reported by Edström and Winblad: probably it takes 2—4 months before the agglutinative reaction becomes demonstrable. At the same time, these 2 serum reactions throw an interesting light upon the connection between rheumatic fever and rheumatoid arthritis.

The studies reported by Dr. Thulin are of particular interest. In the State Serum Institute, Copenhagen, we have tried to reproduce his

findings, but I should not venture to say anything about this question before I am absolutely sure that the methods employed are quite consistent. I think, however, that gradually I am getting to realise that Thulin and I are working with two different agglutinogens. Further investigation will have to show which one is the more serviceable in practice. I should like to hear something about a correlation of the antistreptolysin reaction and Thulin's agglutinin reaction.

According to the results reported here, Thulin's reaction is positive also in acute streptococcal affections (*e.g.* scarlet fever, and angina), whereas this is not the case with the agglutinative reaction employed by Hedlund and me. Furthermore, our reaction seems to keep positive longer than that of Thulin, which falls to negative values rather rapidly, apparently after treatment. I am inclined to think that there is far more direct agreement between the antistreptolysin reaction and Thulin's agglutinative reaction, and I should like to suggest to Dr. Thulin to investigate this problem even further.

O. SIEVERS: In connection with Dr. Hedlund's paper on AST I should like to point out that the outcome of the reaction may vary in intensity depending on the age of the patient. I have examined 141 sera from patients with acute nephritis. Of this total, 49 sera came from adult patients, while 92 came from patients under 16 years. The following results were obtained:

Antistreptolysin titer/ml.

	0—200	201—1000	> 1000	Total
Acute cases	18.4*)	48.9	32.6	141
Adults	24.5	50.9	24.5	49
< 16 yrs.	15.2	47.8	36.9	92

It is a generally recognized fact that children and young persons often react with a stronger antibody response than do adults and this appears to hold true also with regard to the response of the organism to streptococcal lysin. That there is a difference between adults and children in this respect is also evident from Kalbak's studies on healthy subjects. I think that the difference here observed between the result obtained for young and for normal individuals is to be interpreted as signifying a greater capacity of the younger organism for response to the irritation produced by the lysin.

The highest value observed for adult patients with acute nephritis was 2500 units/ml. In the young the corresponding value was 7500 units/ml.; and of the 34 patients in this series who showed over 1000 units/ml. 29 % gave values exceeding 2500 units/ml. On repeated examinations of these young nephritic patients some showed values

*) The figures give the percentage of the total number for each series.

over 8000. In estimating such high values for the reaction, however, the age of the patient should always be taken into consideration.

S. WINBLAD: In a fairly large material of agglutination tests positive reactions were found preponderantly only in rheumatic affections. The method employed in the Malmö Laboratory has been quite in keeping with Kalbak's method, and thus it involves a test for »surface antigen« of hemolytic streptococci. This material originates from two large rheumatic hospitals and from two medical clinics. At the last tabulation, of 467 cases of rheumatoid arthritis 65.1 % were positive (titer of 1:40 or higher). These figures are somewhat lower than given before, but naturally diagnostic variations are conceivable. The absolute value is not of decisive importance, however, as this very well may increase with a more elaborate technique. But the fact that this agglutination occurs mostly in rheumatoid arthritis is of significance, especially theoretical.

In 129 cases of rheumatic fever 41.1 % were found to give a positive agglutination reaction, 58.9 % negative. Besides recent cases of rheumatic fever, however, this material includes also more advanced cases, and no surface agglutination occurs among the recent cases — in contrast to the O agglutination, which seems to appear earlier.

It is of some interest, however, that the agglutination titer in some degree tends to run parallel with the sedimentation rate — as illustrated in Table 1.

Table 1.

Agglutination	Sedimentation rate		
	0—20	21—50	>50/mm/hour
Negative	51	27	12
1/20—1/80	35	32	17
1/60—1/640	24	38	33

In rheumatic fever, as already mentioned, the agglutination titer turns out positive, or increases during the period following the acute stage. I have had occasion previously to demonstrate such cases, and later I have repeatedly been able in the subsequent course of the disease to observe this appearance of the agglutination, which was absent at the acute stage of the disease, when the antistreptolysin titer was positive. Table 2 illustrates this distribution of the occurrence of agglutination among recent and more protracted cases of rheumatic fever.

Table 2.

Time after onset of rheumatic fever	Agglutination		
	Neg.	1/20	1/40 or more
1—3 months	36	7	8
4—6 »	24	4	13
7—10 »	12	3	17

It is to be emphasized that many cases of rheumatic fever show no positive agglutination whatever even though the disease takes a protracted course. In the cases acquiring a positive agglutination, however, it usually appears after the third month of illness.

Naturally it is difficult to say why here we are faced by an antibody that appears late and in markedly chronic cases. My previous opinion — that this would be a question of a necessarily very protracted contact between bacterium and tissue — may hardly be correct. It seems more likely to involve an antigen that is not accessible in the fresh and toxic state of the bacterium, and which manifests itself in this way only when the bacterium has been under the influence of some agency. Possibly further antigenic studies may offer an adequate explanation.

T. PACKALÉN: It would be interesting to learn whether Dr. Thulin in the patient sera which agglutinated antigen A₁ (I, V, XII) but not A (I.....) had tried if the agglutination effect remained after absorption with the latter antigen. According to the antigenic schema, no absorption should take place in these sera.

Further, I should like to ask: Has there been any serum at all that reacted with A but not with A₁?

Finally, I am somewhat surprised at the rapidity with which the agglutinative titer has increased in several of the cases of acute tonsillitis — judging from the diagram, as early as on the third or fourth day after the onset of illness.

The interesting observation reported by Dr. Winblad — about the agglutinative titer rising with increasing sedimentation rate — gives rise to the question, whether perhaps not the same, entirely unspecific, change in the serum that give rise to the increased tendency to aggregation of the red blood cells — as manifest in the increased sedimentation rate — might not also increase the tendency of the streptococci constituting the antigen employed to agglutination under the influence of specific antibodies — and thus increase the titer.

K. E. THULIN: The reactions in acute tonsillitis show, I think, that here we meet with an acute antibody that appears relatively early in the course of the disease. Among the four cases, in one the agglutination took place immediately at the onset of illness, in two on the sixth day of illness, and in one about two weeks after the onset.

The relatively early attainment of such high titer values may perhaps in part be attributable to the common occurrence of streptococcal infection. Presumably repeated attacks of tonsillitis may permanently stimulate the antibody formation, so that in certain cases an increased titer may be expected relatively early in the course of the disease, as the O agglutinin titer often may remain for a couple of months. Something similar applies also to the antistreptolysin reac-

tions, and hence it may be of interest to compare the AST values and the O agglutinin values.

In order to throw some additional light on this question, at present some investigations into this matter are being carried out in collaboration with Dr. Kåre Berglund, Military Hospital, Stockholm. In cases of acute nephritis, AST determinations are made parallel with streptococcal O agglutinations. From the material collected so far — about 20 cases of acute nephritis — it looks as if there is a distinct connection between the two antibodies concerned, with partly parallel shifts in the antibody titers.

In contrast to AST, however, the O agglutinin content remains constant on the transition from acute to chronic nephritis.

The divergence of reactions between antigens A_1 and A was seen in 5 cases; 4 cases gave a positive reaction only with antigen A_1 , one positive with antigen A.

Of course, Professor Packalén is right in pointing out that, as antigens A_1 and A both contain partial antigen I, while A_1 also contains V and XI, the reaction ought to be obtained with partial antigen V or XI in sera that are positive only on agglutination with antigen A_1 . Indeed, some of these sera have been found to agglutinate partial antigen V to a titer of 1:40, but this titer is all too low to allow of any definite conclusions. Experiments on this question are being continued, however, so that perhaps it may be elucidated better.

EXPERIMENTS ON ACTIVE AND PASSIVE PERMEABILITY OF BACILLUS COLI COMMUNIS*)

By Søren L. Ørskov.

(Received for publication August 28th 1947).

Very little is known of the permeability of bacteria with the exception of *Hofmann* and *Ruhland's* experiments (1925) on *heggiatona mirabilis*. These bacteria cannot be plasmolysed in hypertonic solutions.

Fisher (1891) investigated the permeability of different bacteria by following the disappearance of plasmolysis in hypertonic solutions. Some substances permeated so fast, that deplasmolysis could not be followed.

Three methods have been used in my experiments in order to follow the plasmolysis and deplasmolysis in hypertonic solutions.

- 1) Ordinary cover-slip-slide preparations from the suspensions of the bacilli.
- 2) An apparatus for continued examination of suspensions which is demonstrated at this congress. (*Ørskov* 1947.)
- 3) A photoelectric method, published 1935, (*Ørskov* 1935¹ and *Ørskov* and *Meldahl* 1940) which I have used to examine the permeability of red blood cells and yeast cells (1946). The principle of the method is that bacteria, which are plasmolysed show a decreased transillumination.

I shall begin my report by giving some figures from potassium analyses on coli bacilli, which had been placed in a hypertonic sodium chloride solution.

The coli bacilli were cultivated in peptone-bouillon for 20 hours

*) This paper was read at the 17. International Physiological Congress, Oxford, 1947.

at 37° and then centrifuged at 3000 revolutions per minute after which the bacilli were suspended in a little of the culture, so that the suspension contained about 10 vol. % bacilli, ascertained by centrifugation in haemotocrit tubes at 10,000 revolutions per min. for 10 minutes.

1.5 cc of the suspension was then mixed with the same volume of 0.9 % NaCl as a control and other samples with 3.04 % NaCl, so that the osmotic pressure here was doubled.

After the lapse of various times these samples were centrifuged by 10,000 revolutions per minute for 10 minutes, the supernatant fluid was sucked away, the glasses twice cautiously filled with distilled water, which was afterwards removed. Then the bacilli were transferred to crucibles, ashed, and the potassium determined.

As seen from table 1 potassium of the bacilli in the hypertonic solution is increased 31—68 %.

The concentration of potassium in the extracellular fluid is about 50 mg %, the concentration in the bacilli about 400 mg % so that the absorption of potassium undoubtedly involves an active process. The volume of the cells measured by haematocrit determinations is but little reduced in hypertonic solutions. By microscopic determinations you will find that the bacilli in the hypertonic solution at first show a pronounced plasmolysis, which after a few minutes decreases, but in most cells some plasmolysis remains.

Potassium is concentrated in the cells by an active process as can be shown by adding enzyme inhibitors.

Table 1.
Potassium in mg % of bacilli suspended in bouillon culture.

0.9 % NaCl	Minutes after 1.97 % NaCl						Max. % increase
	4	7	13	30	60	120	
384				560			46
393			660				68
316		452					43
506	713						41
397					635		60
322						487	38
440			537			577	31
<i>1.97 % NaCl + 0.1 % NaN₃ in the suspension</i>							
238			258	263	226	236	10
402			460	458			14
403			433	467			16
<i>0.5 % glucose in the suspension</i>							
352			432	425	396	337	23

From table 1 it appears that sodium azide hampers the process to a high degree, and moniodo-acetic acid and hydrocyanic acid have the same effect, as has been ascertained by microscopic examinations. Na F and urethane are of little or no influence.

As mentioned the plasmolysis is not quite abolished by absorption of potassium. If you make the experiments so that the suspensions of the bacilli are not so concentrated, if you for instance use the original culture and add the same volume of 3.04 % NaCl one will see that the plasmolysis disappears completely very soon, in some cases so fast that the process has finished before one can examine the suspension under the microscope.

It has not been established if under these circumstances the potassium absorption is so great that it can explain the deplasmolysis.

However, there is no doubt that deplasmolysis can be caused by other substances than potassium. If one in the experiments with hypertonic solutions of NaCl add a little glycerol, plasmolysis will disappear. This is probably not caused by a stimulation of potassium absorption. Glycerol permeates extremely fast causing no plasmolysis at all.

Probably glycerol in the cells is converted into osmotically active substances, which cannot leave the cells.

Addition of glucose will also make deplasmolysis perfect, and can do so even if the concentration of NaCl in the surrounding fluid is 5—6 %, but then it seems as if many cells burst, at any rate the haematocrit value are $\frac{2}{3}$ to $\frac{1}{2}$ of the controls though the remaining cells look quite normal.

Glucose has previously been shown to give rise to a potassium absorption of the same dimensions as shown in these experiments in hypertonic solutions.

As in these experiments maximal absorption is reached after a few minutes, but in the glucose experiments potassium absorption is very soon followed by potassium loss. Thus, in some experiments, after 1 hour less potassium is found in the cells than before the experiments.

In table I such an experiment is shown with 0.5 % glucose in the suspension.

Pulver and Verzar (1940) first showed that yeast absorbed potassium when glucose was added. They think that potassium is bound when glycogen is synthesized in the cells, and is lost again, when the glycogen is broken down.

Leibowitz and Kupermintz (1942) find that coli bacilli absorb potassium after addition of glucose and that the absorption is maximal after 5 minutes.

Conway and Malley (1946) think that potassium is absorbed through exchange of potassium ion with hydrogen ion.

Rothstein and Enns (1946) find that glucose-absorptions can proceed without potassium in the medium and at the same rate. Like *Verzar* they find that the process can be stopped by enzyme paralyzing

poisons. They find in most cases potassium absorption and production of acid to be of the same magnitude.

It can be shown that potassium absorption can go on even if the organic substances in the medium are much deluted. In table 2 is shown an experiment where the centrifuged bacilli are suspended in a solution containing 0.65 % NaCl and 0.19 % potassium chloride.

Table 2.
Potassium in mg % of bacilli suspended in 400 volumes 0.65 % NaCl and 0.19 % KCl.

0.9 % NaCl	Minutes after 1.97 % NaCl				Max. % increase
	13	30	60	120	
272	329	368	336	324	35
	<i>Suspended twice in the same solution</i>				
408	443	425			7
420		454			8

After addition of hypertonic NaCl solution the cell potassium is augmented by 35 %.

If the cells first are washed in a large volume of the above mentioned solution (400 times the cell volume), potassium absorption is much diminished or has ceased possibly because chloride is only absorbed with difficulty and no other acid is found in the solution. Probably 8 % increase means that no potassium absorption has taken place as the cells are plasmolyzed and the fluid between the protoplasm and the cell membrane contains about 50 mg % potassium.

Table 3.
Loss of potassium in 0.9 % NaCl.

	mg % K.	
Bacilli from bouillon culture	376	
	370	
Bacilli washed once in 70 volumes 0.9 % NaCl	310	K decreased
	317	16.5 %
Bacilli washed twice in 70 volumes 0.9 % NaCl	262	K decreased further
	254	18 %

If coli bacilli are suspended in 0.9 % NaCl they lose potassium. In table 3 the first experiment shows the potassium content of bacilli, centrifuged from a bouillon culture. The next shows the potassium content after the bacilli have been washed once in 0.9 % NaCl (70 × the volume of the bacilli). In the last experiment the bacilli have been washed twice in 0.9 % NaCl.

Each washing gives a loss of about 17%, which is partly caused by intercellular fluid, but partly by a loss of potassium from the cells.

If one examines twice washed bacilli under the microscope one finds that most of them are plasmolysed. The plasmolysis will disappear if you add potassium chloride and glucose or glycerol.

The potassium absorption of the bacilli in hypertonic NaCl solution is no doubt appropriate. Plasmolysis gives a dead space between the outer cell membrane and the protoplasm which impedes diffusion to and from the protoplasm and probably the functions of the cells are also impeded.

Possibly is it a regulation which is found in many kinds of cells — especially if the cells must live at changing osmotic pressures of the surrounding medium or if the osmotic pressure of the cells changes.

It has still not been mentioned how I imagine that the acids, which must follow potassium, pass into the cells. From experiments with the photoelectric method it has been concluded that chlorine ions only with difficulty permeate the protoplasm. Ammoniumchloride does not permeate or only very slowly, but ammonium acetate permeates so fast that no plasmolysis will occur. It is assumed that permeation of ammonia and acetic acid takes place easily. No experiments have been made to show if carbon dioxide is of any importance to the absorption of potassium, but the author has shown in previous publications that ammonium salts permeate much faster through the blood cell membrane, when carbon dioxide is present and so does potassium, when the cells are poisoned with lead (*Ørskov* 1933, 1934 and 1935).²⁾

Conway and *Breen* (1945) have shown that potassium of yeast cells can be exchanged with ammonium, when carbon dioxide is present.

Coli bacilli are easily permeable to lipoid soluble substances, and as there are many organic acids in bouillon the permeation of the acids need not be a problem. But how potassium is absorbed I don't know. It is possible that potassium ion according to *Conway* is exchanged with hydrogen ion, but still the mechanism is not very clear.

A question which arises is whether the potassium absorption in glucose solutions and in hypertonic NaCl solutions are two different regulations or if they at any rate partly have the same aim.

An absorption of potassium in glucose solution would be appropriate as it would be able to neutralize part of the acids formed in the cells and keep them in the cells. But there may also be another explanation of the absorption. If osmotically active substances were bound in the cells when glucose absorption starts, then the osmotic regulation would begin a potassium absorption. It should be possible to show if the osmotic pressure during glucose absorption is changed.

These experiments were started in order to study the permeability of the *coli* bacilli with the photoelectric method. With this method it

has been established that some substances, when added as a hypertonic solution pass so fast into the cells that no plasmolysis takes place, for inst. Alcohol, urethane, hexamethylenetetramine, glycerol and antipyrine. This is in accordance with the experiments of *Fisher* (1895). Urea passes at a slower rate, and the plasmolysis caused by malonamide will only have disappeared after 6—8 minutes. If there is no potassium in the solution, substances like mannite, glucose and arabinose will pass very slowly into the cells, and deplasmolysis will be very slow. But if there is potassium in the solution, (and only very little potassium is needed) this ion will be absorbed, partly because the substances are sugars, partly because the osmotic pressure has been increased and deplasmolysis sets in and so simulates the permeation of the added substances.

The consequence is that such permeability experiments with coli bacilli must be carried out with cells washed in 0.9 % NaCl in order to avoid the osmotic regulation, as sodium does not seem to permeate, and the active processes of the cells must perhaps be paralyzed by enzyme inhibitors.

But then there is the possibility that this treatment will have changed the characteristics of the protoplasm and at any rate the cells will be plasmolysed on account of potassium loss.

Summary.

1) By direct microscopic examination and by a photoelectric method it is shown that some substances when added in hypertonic solutions permeate so fast that no plasmolysis of coli bacilli occur. f. inst. alcohol, glycerol, hexamethylenetetramine and antipyrine.

2) With urea the deplasmolysis has taken place after 1—2 minutes, with malonamide after 6—8 minutes and with these substances rather constant values are found.

3) In hypertonic solutions of glucose, fructose and mannite deplasmolysis will occur very slowly, when the bacilli are thoroughly washed with 0.9 % NaCl.

If potassium is present in the suspension or in bouillon culture deplasmolysis is finished after a few minutes.

It has been shown by *Pulver* and *Verzar* (1940) for yeast and by *Leibowitz* and *Kupermintz* (1942) for coli bacilli that the addition of glucose gives rise to a considerable potassium absorption.

4) When a suspension of coli bacilli in bouillon is mixed with a hypertonic sodium chloride solution plasmolysis will have disappeared after a few minutes. Even in a 5—6 % NaCl solution many cells will look normal if glucose is added to the solution.

5) It is shown that deplasmolysis to a great extent is caused by potassium absorption.

If the osmotic pressure of the bouillon is doubled by adding NaCl, potassium of the cells is increased 31—68 %.

6) The potassium absorption is an active process as the potassium concentration of the cells is much higher than the concentration of the fluid outside the cells.

The potassium absorption in hypertonic sodium chloride solutions can be hampered to a high degree by sodium azide, hydrocyanic acid and moniodo-acetic acid. Sodium fluoride and urethane have little effect.

7) If coli bacilli are thoroughly washed in a solution containing 0.65 % NaCl and 0.19 % KCl, the addition of hypertonic NaCl solution will cause no potassium absorption, probably because no weak acids are present to follow potassium into the cells, as chlorine ion seems to permeate only with difficulty.

8) The appropriateness of the established osmotic regulation and its relation to potassium absorption in glucose solutions is discussed.

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EXPERIMENTAL INVESTIGATION INTO THE GENESIS OF THE ADRENOGENITAL SYNDROMES BY ANALYSIS OF THE MORPHOLOGY OF THE PITUITARY IN HYPER- AND HYPOFUNCTION OF THE ADRENAL CORTEX IN RATS*)

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As is known, certain diseases with genital disturbances are characterised by enhanced function of the adrenal cortex, and have therefore been called adrenogenital. Cushing's disease and certain forms of virilism are undoubtedly in this category. Morgagni's syndrome, which is so common among women in the menopause, and which in its complete form presents adiposity of the torso, hirsutism and frontal enostoses, has also rightly been included here, primarily due to its resemblance to Cushing's disease, both in externals and in the occurrence of hyperplasia of the adrenal cortex. It is possible that hypertrophy of the prostate is also linked up with hyperfunction of the adrenal cortex (Mellgren 1945).

In earlier works (1942, 1945) the present author has shown that all the diseases mentioned are characterised by the same histological change of the anterior pituitary, namely an increased number of hyaline basophiles — first described by Crooke (1935) in Cushing's disease — together with an increased number of hypertrophic amphophiles, i. e. large chromophobe cells with hypertrophic nucleus — and often of sparsely granulated basophiles also. The change in the pituitary appeared regardless of whether the adrenal cortex in the individual case was the seat of adenoma, cancer, or merely hyperplasia. On the other hand, it was quantitatively largest in Cushing's disease and virilism.

*) Given in a shortened form as a lecture at the meeting of the Scandinavian Pathological Association in Uppsala 7—9 July 1947. — The expenses of the investigation have been defrayed by grants from the University of Lund.

The same work (Mellgren 1945) evinced a statistically significant, positive correlation between the frequency of the mentioned pathological pituitary cells and the size of the adrenal cortex. This correlation was still significant in a material of endocrinely normal subjects + cases of Morgagni's syndrome + cases of prostatic hypertrophy (about 75 individuals) — that is to say, where the extreme cases (Cushing's disease and virilism) had not been included. No regular change in any other endocrine organ could be shown to explain this correlation. The logical conclusion is *either* that the hyperfunction of the adrenal cortex induces the pituitary change described, or, conversely, that the pituitary change indicates an overproduction of corticotrophic hormone, which induces the adrenal disturbance.

The main problem of the present work is to discover which of these alternatives is the right one. Naturally, in either alternative it is possible that the quantitatively increased hormone production — from the adrenal cortex and the anterior pituitary respectively — is also of pathological quality, but this is a question which must for the present be shelved.

Earlier experimental and clinical observations have already suggested that the second alternative — i. e. overproduction of corticotrophic hormone and secondary hyperfunction of the adrenal cortex — is what actually takes place in the adrenogenital diseases; this probably holds in cases of tumours of the adrenal cortex also.¹⁾ Hodler (1936, 1937) showed the possibility of this by including simultaneously hyperplasia of the adrenal cortex and virilism in castrated female guineapigs by injections of corticotrophic pituitary extracts. Several authors have furthermore induced adenoma in the adrenal cortex with pituitary extracts (Putnam *et al.* 1929; Anselmino *et al.* 1933, 1934, 1935) and, in a definite mouse race, Woolley and co-workers (1943) induced adrenal cortical cancer by means of gonadectomy, the operation affecting the adrenal cortex probably via the anterior pituitary. Further, Jores and co-workers (Jores and Beck 1935; Jores 1936; Brauer 1937; Jacobi and Tigges 1939; Sundermann 1940; Berblinger 1940, 1943) found an increased content of corticotrophic hormone in the blood in several cases of Cushing's disease and in one case of virilism. The testing method used is hardly convincing, however.

On purely morphological grounds, too, a good case can be made for saying that the pituitary change in the adrenogenital syndromes implies overproduction of corticotrophic hormone. That is to say, all the pathological pituitary cells which are found are in all probability developed from basophiles: moreover, their considerable size and large, loose nuclei with big nucleoli indicate a strong function (Mellgren 1945). Besides this, Berblinger (1943) has clearly shown that the basophiles are carriers of the corticotrophic hormone. On the

¹⁾ For a full survey of the older literature, see Mellgren, 1945.

other hand, Ingle (1938) has shown that injections of large doses of cortin is followed rapidly by atrophy of the adrenal cortex in normal rats, but not in hypophysectomised ones. Another possibility to be allowed for, therefore, is that the strong activity of the basophile pituitary cells in the adrenogenital diseases may indicate a depressing action on the adrenal cortex, which it would be reasonable to assume secondary to the cortical hyperfunction.

Finally, a comparison with the pituitary picture in Addison's disease provides certain criteria when assessing the genesis of the adrenogenital syndromes. It is true that the pituitary in Addison's disease shows certain special features, such as a reduced number of basophiles, increased number of chromophobes, and — sometimes — few acidophiles (Kraus 1923, 1927; Hewer 1923; Guizzetti *et al.* 1928; Berblinger 1932, 1942; Harrop *et al.* 1932; Terplan and Sanes 1932; Lang 1946); the same frequency changes have often been established by a differential count (Crooke and Russell 1935; Hawking 1936; Mellgren 1945). Only in a few isolated cases of Addison's disease has a sure frequency increase of the pituitary basophiles been observed (Severinghaus 1938). However, most cases of Addison's disease have also shown cytological changes in the basophiles, indicating increased activity (cf. Severinghaus 1938) resembling that in the adrenogenital syndromes. Thus, finds have been made — particularly in slight cases of Addison's disease — of numerous enlarged, sparsely granulated basophiles (Berblinger 1932, 1942; Terplan and Sanes 1932; Crooke and Russell 1935; Severinghaus 1938; Mellgren 1945), and also a coalescence of the basophile granules into homogeneous or hyaline masses (Kraus 1923, 1927; Berblinger 1932, 1942; Crooke and Russell 1935; Mellgren 1945), which gives the cells a certain resemblance to Crooke's hyaline basophiles. In addition, in 1945 the present author found typical hyaline basophiles and hypertrophic amphophiles in definitely increased numbers in one case of two with unquestionable Addison's disease (adrenal tuberculosis).

A recently observed case of Addison's disease is also worthy of mention:

Tmb. 4740/47, Lund. (Clinical examination and autopsy at Karlskrona hospital, Dr. L. Lundgren). 17-year-old woman. Addison's disease, beginning acutely with angina in September 1945. Reacted promptly to desoxycorticosterone acetate (DOCA) given intramuscularly. From March 1946 DOCA tablets subcutaneously. In December 1946 the blood sugar suddenly rose to 500 mg/100 cc, and later to 800 mg/100 cc, and acidosis set in.

Insulin sensitivity: 8 + 8 internat. units gave normal blood sugar values. In June 1947 acute fever and headache, picture of encephalitis.

L. R.: 14 mononuclear cells/1 mm³, protein reactions negative. Patient did not eat. Died from general cachexia.

Microscopic examination (Mellgren) showed the following in addition to signs of sepsis: The fat from the *adrenal region* contained on one side a small fragment of an adrenal: the cortical cells were small and poor in lipid, lying few and far between in abundant connective tissue with sparse infiltra-

tion of round cells. The *pancreatic islands* were small, with atrophic lymphocytelike cells. The *pituitary* was macroscopically normal. The general histological structure of the anterior lobe was normal, no adenomas, no fibrosis. Acidophiles fewer than normal, small. Normal basophiles extremely few (at an estimate some $\frac{1}{100}$). Numerous basophiles almost free from granules, with large body and large, loose nucleus. Many basophiles with granules merging into hyaline seales, and with a large loose nucleus with large nucleolus; some of these cells were confusingly like Crooke's hyaline basophiles. More chromophobes than anything else; many were large, with a gigantic nucleus and large nucleolus — i. e. like the hypertrophic amphophiles in adrenogenital syndromes.

Thus, in this case of Addison's disease also, the anterior pituitary showed signs of strong basophilic activity, very like that in the adrenogenital diseases. Now the pituitary changes in the two quoted cases of Addison's disease cannot have arisen from hyperfunction of the adrenal cortex; on the other hand, it might be possible to trace the similar pituitary changes in Cushing's and Addison's disease to a raised corticotrophic pituitary function common to both diseases.

The cases of Addison's disease which have been systematically compared with the adrenogenital syndromes are, however, too few to allow of general conclusions. In this situation, the present author has tried by experiment to throw light on the genesis of the pituitary change in adrenogenital syndromes by investigating the state of the pituitary in rats both after induced hyperfunction of the adrenal cortex and after adrenalectomy.

Experiments into hyperfunction of the adrenal cortex and its effect on the pituitary have admittedly been made earlier on, but the results contradict one another. Different effects on the anterior pituitary have been reported by different authors after the injection of adrenal cortex extracts (Lippross 1936; Jores 1937—38; Heiden and Kahlau, *cit.* Romeis 1940). This is natural, as the extracts were not pure.

A clue for better procedure can be obtained from Hodler (1936, 1937), as mentioned above. The virilism which, together with growth of the adrenals, she induced by the injection of pituitary extracts into castrated female guineapigs, was not forthcoming after adrenalectomy. With pure corticotrophic hormone, therefore, it should be possible to induce an adrenal cortex hyperfunction similar to that in virilism, and to study its reaction on the pituitary.

An experiment of this kind has been performed by Koneff (1944). He gave male rats pure corticotrophic hormone according to Evans and co-workers in large doses for up to 30 days. The pituitaries become lighter; the only histological changes were in the basophiles, which grew smaller and developed pyenotic nuclei, small Golgi apparatuses, and lost some of their granules. The changes were established by planimetry, but no differential count of the cells was made. Koneff rightly maintains that the appearance of the change clearly argues

reduced basophilic activity. That this reduced activity corresponds to a reduced excretion of corticotrophic hormone from the pituitary can be deduced from Ingle and Kendall's experiment (1937), which showed that raised function of the adrenal cortex inhibits the pituitary's excretion of this hormone. With the time of treatment, and in the large doses which Koneff gave the rats, Evans's corticotrophic hormone also induces retardation of other pituitary functions, however, which dims the significance of the pituitary change; Ewans and co-workers, using the same hormone in similar experiments, obtained retarded body growth (Ewans, Simpson and Li 1943), atrophy of the thymus and lymph nodes (Simpson, Li, Reinhardt and Evans 1943), reduced development of the testes and the accessory sex organs, together with loss of weight and some histological hypoplasia of the thyroid (Koneff 1944).

Experiments with adrenalectomy. As regards the effect of adrenalectomy on the function of the anterior pituitary, Faber (1945) showed that the operation on rats is followed after no more than 5—8 days by an increase of the lobe's content of corticotrophic hormone. Collip and others (Collip, Anderson and Thomson 1933; Shumacker *et al.* 1934) showed that the operation also caused raised concentration of corticotrophic hormone in the blood; after unilateral adrenalectomy these investigators obtained the usual compensatory growth of the other adrenal, but *only* in normal animals, not in hypophysectomized ones.

This makes it likely that, after adrenalectomy, the anterior pituitary also produces more corticotrophic hormone than normal. The present author has assumed this to be so, as a working hypothesis, well aware of the less probable possibility that the increased concentration in the anterior pituitary and the concurrently raised concentration of corticotrophic hormone in the blood might be due to reduced consumption in the periphery.

The earlier literature is confusing and contradictory on the morphological effect of adrenalectomy on the anterior pituitary, due partly no doubt to the short period that the experiment animals survive, and partly to the absence of a differential count of the pituitary cells (Igura 1927; Lehmann 1929; Martin 1932; Nicholson 1936; Herrick *et al.* 1940; cf. also Shumacker and Firor 1934, and Berblinger 1942). Greater interest attaches to the experiments into chronic adrenal insufficiency performed by Grollman and co-workers (Shumacker and Firor 1934; Grollman and Firor 1935). They gave completely adrenalectomized dogs and rats small, inadequate doses of adrenal cortex extract, which just kept the animals alive up to 100 days. During this time the animals showed a constant body weight, lowered body temperature, atrophy of the genitals and thyroid, and hypoplasia of the thymus and lymph nodes. 100 days after the operation the pituitaries of the dogs showed few or no basophiles; those of the rats showed

some decrease of the basophiles, some of them abnormally tingible. The change is said to resemble that in Addison's disease, but is not described in any detail. No differential count was made.

Author's own experiments

Material and methods. The experiments cover 147 young fully grown white rats of the same heterozygote strain. To avoid influence of the sexual cycle on the pituitary picture, mainly males were used (128 animals); only complementary experiments, which were dealt with separately, used females also (19 animals). The experiment animals and controls were throughout divided up in such a way that animals of the same weight from the same litter were always in different groups. The groups were then compared with one another as entities, always taking into account the age of the animals (see below).

From one week before the experiments began, the animals were kept at a relatively constant room temperature of $+18^{\circ}$ — $+21^{\circ}$ C, and fed on a special animal bread¹⁾ *ad lib.*, water *ad lib.*, and about 5 cc of milk per animal daily. Each animal was weighed every day.

Hypophysectomies were performed according to Collip, Selye and Thompson (1933) with avertin narcosis. Only when the operation was technically perfect were the animals used. Animals which after the operation lost much weight, or seemed ill or dead, were also rejected. At the end of the experiments the completeness of the operation was checked with the microscope, and animals with pituitary remains were thrown away.

Adrenalectomies were made by Bomskov and Bahnsen's method (1935), with ether as the narcotic, though avertin was used when this operation was performed at the same time as hypophysectomy. Animals dying within 9 days of the operation were thrown away. At the end of the experiments the completeness of the operation was checked histologically.

At the end of the experiments the animals were swiftly decapitated without narcosis far down on the neck, so that the thyroid gland should not be injured. The pituitary, thyroid with parathyroids, adrenals, testes (ovaries), seminal vesicles, prostate (uterus) and kidneys were prepared and weighed. All the organs except the pituitaries were fixed in 10 % formalin. Frozen sections were made of the adrenals through the centre, which were stained both by the Schmidt-Dietrich method and with Scharlach R; other organs were embedded in paraffin and stained with eosine.

The pituitaries were fixed, sectioned in series horizontally, and stained with buffered eosine and aniline blue solutions, all by Mell-

¹⁾ Prepared by Konsum, Stockholm, according to S. Gard's recipe: 100 kg milk, 53 kg unseasoned breadcrumbs, 20 kg groats, 15 kg wheat sprouts, 10 kg Lucern hay meal (*Medicago sativa*), 2.5 kg fish meal, 40 gm cod liver oil. Mix, and dry at 40° C.

gren's method 1944, and a differential count made according to Mellgren 1945, with certain modifications. The following six cell types were registered in this way: acidophiles, normal basophiles, normal chromophobes, pycnotic basophiles, hyaline basophiles (including both typical Crooke cells and basophiles with granules coalesced into small hyaline scales), and hypertrophic amphophiles (defined as large chromophobe cells with nuclei over 7.6×7.6 or $10.2 \times 5.1 \mu$). That is to say, the limit for the minimum size of cell nucleus in the hypertrophic amphophiles has been placed somewhat lower in the rats (broadly speaking, a minimum of $45 \mu^2$ in the plane of the section) than in human subjects (minimum $50 \mu^2$, Mellgren 1945) since the pituitary cells in rats are normally smaller. In this way, a differential count of about 2000 cells was made for each animal in a horizontal section near the centre of the pituitary.

Preliminary experiments determined the exactitude of the counting method in the modification mentioned. First, a differential count was made of a horizontal section near the centre of the pituitary from a normal male rat according to Floderus' method (Floderus 1944). This showed a fairly even distribution of the different cell types in different parts of the slide, so that 2000 counted cells was deemed sufficient. In another experiment the effect of varying levels in the horizontal section was determined as follows. First of all, a differential count was made of about 2000 cells in a horizontal pituitary section about 60μ above the centre, and the process was then repeated in another section about 60μ below the centre of the same organ. This gave two values (in per cent) for the frequency of each cell type, with a difference, d . The experiment was repeated with 10 animals from different groups. The dispersion of a single determination (σ_i) was then calculated according to the usual formula for double determinations, $\sigma_i = \pm \sqrt{\frac{\sum d^2}{2N}}$. The result is shown in table 1. As is seen, the dispersion is small, despite the great difference in the levels of the investigated sections.

Table 1.

Dispersion of a single determination (σ_i).

Calculated on the difference between two separate horizontal sections (d) for each case.

Cell types	Number of investigated cases N	Cell frequency in % Mean of all cases M	Dispersion of single determination in % σ_i
Acidophiles	10	35.8	± 1.9
Normal basophiles	10	13.8	± 1.0
Normal chromophobes	10	47.9	± 2.0
Pycnotic basophiles	10	1.4	± 0.2
Hyaline basophiles	10	0.2	± 0.1
Hypertrophic amphophiles	10	1.0	± 0.2

When *organ weights* of any one experiment group were compared with those of normal animals, the ages of the individual animals were taken into account according to the following method, suggested by Prof. C. E. Quensel, Lund. First of all each organ of the normal animals was given a straight regression line according to the equation $y = a + bx$, where y is the weight of the organ in mg, x is the age in days, and a and b constants, calculated in the usual way. The average perpendicular deviation (D) from this regression line was then determined for the corresponding organ weights of the experiment animals according to the formula $D = m_y(1) - a - b \cdot m_x(1)$, where $m_y(1)$ is the mean of the experiment animals' organ weights, and $m_x(1)$ that of their age. Provided that the experiment animals' regression line is parallel to that of the normal animals, the standard error of D has been calculated (allowing for the standard error of a at $m_x(1)$) according to the formula

$$\epsilon^2(D) = \frac{\mu^2(1)}{n_1} + \frac{\mu^2}{n} \left[1 + \frac{(m_x - m_x(1))^2}{\left(\frac{\sum x^2}{n} - m_x^2 \right)} \right]$$

where $\pm \epsilon(D)$ is the standard error of D , n the number of normal animals and n_1 the number of experiment animals, m_x the mean of the normal animals' ages, $m_x(1)$ the mean of the experiment animals' ages, $\sum x^2$ the sum of the squares of the individual normal animals' ages, and μ^2 and $\mu^2(1)$, i. e. the residual squares, have been calculated according to the formulas

$$\mu^2 = \frac{\sum y^2 - a \sum y - b \sum xy}{n - 2}$$

and

$$\mu^2(1) = \frac{\sum y^2 - n \cdot m_y^2(1) - 2b(\sum xy - n_1 m_x(1) \cdot m_y(1)) + b^2(\sum x^2 - n_1 \cdot m_x^2(1))}{n_1 - 1}$$

where $\sum y^2$ etc. refer to the normal animals, and \sum, y^2 etc. to the experiment animals.

When making corresponding calculations for the *body weights* of normal and experiment animals, it was found more suitable to use logarithmic regression, and so x was made to indicate log age in days.

Animals which were hypophysectomized and then treated with different hormone doses were, together with their controls, selected from within narrow age limits. It was therefore not necessary to use the above statistical method for these groups; instead, correlation coefficients (Bravais-Pearson) were calculated between weights and the size of the doses according to the formula

$$r = \pm \frac{(n-1)}{n} \cdot \frac{\left(\sum xy - \frac{\sum x \cdot \sum y}{n} \right)}{\sqrt{\left(\sum x^2 - \frac{(\sum x)^2}{n} \right) \left(\sum y^2 - \frac{(\sum y)^2}{n} \right)}}$$

Experiment 1. Control testing of the corticotrophic hormone, to be used in the main experiment. The hormone (stated to be crystallinely pure) had been prepared according to Max Reiss's method, and placed at my disposal by him through the kind offices of Pharmacia and Dr. F. Paulsen. The hormone was stated to contain 10 unit/mg. Reiss *et al.* (1936) define 1 unit as the smallest quantity which obliterates the sudanophobic zone of the adrenal cortex in 2 out of 3 hypophysectomized rats weighing 80—120 gm, the quantity being divided into 16 doses, of which 2 are given daily for 8 days.

The hormone was tested out on 3 groups of hypophysectomized animals thus:

Group A: Testing mainly according to Reiss *et al.* 1936. 7 rats were used, 4 females and 3 males, aged 68—119 days. Hypophysectomy was followed 12—16 days later by left-sided adrenalectomy; hormone injections were begun at the same time: 1 dose in 0.2 cc of 0.9 % saline subcutaneously twice daily for 8 days. Dosage: 1 ♀ given 0.06 mg, 1 ♂ and 1 ♀ 0.02 mg, 1 ♂ and 1 ♀ 0.006 mg, 1 ♀ 0.002 mg and 1 ♂ 0 mg per dose. 12 hours after the last injection all the animals were killed.

No effect from the hormone could be demonstrated: the cortex of both adrenals showed an equally distinct sudanophobic zone in all animals, and the right adrenal was in all cases somewhat lighter than the left.

Group B: Testing according to Reiss *et al.* 1936. 15 females, 103—106 days old, were hypophysectomized. 15—18 days after the operation, a dose of hormone diluted in 0.2 cc of saline was given subcutaneously twice daily for 8 days. Dosage: 3 animals were given 0.17 mg, 2 animals 0.06 mg, 2 animals 0.02 mg, 3 animals 0.006 mg per dose. 5 females, only hypophysectomized, were used as controls. All the animals were killed 12 hours after the last injection.

This group did not show any effect from the hormone, either. The cortex of the adrenals presented an equally distinct sudanophobic zone in all cases. The weight of the adrenals at the sectioning was correlated with the size of the dose given to each of the 15 animals; the correlation coefficient according to Bravais-Pearson was -0.14 ± 0.25 . In the same way, the loss in body weight from hypophysectomy to dissection was correlated with the size of the dose, the correlation coefficient being -0.01 ± 0.26 . Similarly, the coefficient for the correlation between the weight of the thyroid and the dose was -0.23 ± 0.24 , and for the correlation between the weight of the ovaries and the dose -0.19 ± 0.25 . Nor was the weight of either uterus or kidneys changed by the hormone treatment.

Group C: Testing mainly according to Simpson, Evans and Li (1943). 14 males, 45—51 days old, were subjected to hypophysectomy and left-sided adrenalectomy in the same bout. Hormone was given at once, one dose daily in 0.2 cc of saline intraperitoneally, for 16 days.

Dosage: 4 animals were given 3.6 mg, 3 animals 0.6 mg, 4 animals 0.1 mg, and 3 animals 0 mg per dose. They were killed 24 hours after the last injection.

With these doses and testing conditions, the hormone had a definite effect. The sudanophobic zone in the right adrenal was distinct and of varying breadth in the 7 animals receiving 0—0.1 mg of the hormone per dose; it was almost entirely absent in one, and was quite narrow in 2 of the 3 animals receiving 0.6 mg/dose; it was completely absent in the 4 animals given 3.6 mg/dose. The left adrenals showed no sudanophobic zone. The correlation coefficient according to Bravais-Pearson between the breadth of the sudanophobic zone in the right adrenal (divided into 4 classes, 0—4) and the size of the dose given was -0.83 ± 0.08 for the 14 animals.

In all 14 animals, the right adrenal was found to be lighter than the left. The difference in weight between left and right adrenal was calculated (*ad modum* Collip 1933, cit. Bomskov 1939) for each animal in per cent of the weight of the left adrenal. The correlation between the difference and the hormone dose administered was -0.49 ± 0.20 for the 14 animals. Thus was elicited a certain, though quite faint possibility ($0.05 > P > 0.02$) that the hormone retards the adrenal atrophy after hypophysectomy.

The hormone had no effect on the body weight, the correlation between the loss in weight during the test and the size of the dose turning out to be -0.13 ± 0.26 . Nor was there any effect on the weight of the kidneys (r for kidney-weight — dose: $+0.05 \pm 0.27$) of the thyroid (r for thyroid-weight — dose: $+0.07 \pm 0.27$). On the other hand, the testes became somewhat heavier as the hormone dose increased ($r = +0.51 \pm 0.20$); the same applied to the accessory sex organs, i. e. prostate + seminal vesicles ($r = +0.48 \pm 0.21$). The probability that the hormone really retards the atrophy of the testes and the accessories is therefore very faint ($0.05 > P > 0.02$). Even if the effect on testes and accessories can be regarded as certain, it need not indicate admixture of a gonadotrophic factor in Reiss's hormone, but may be a result of this hormone's corticotrophic effect — Selye and co-workers (1940) obtained definite gonadotrophic effect from desoxyzycortio-sterone on hypophysectomized rats.

Summing up, we can say that the Reiss hormone which was used showed unquestionable corticotrophic («anti-sudanophobic») effect on hypophysectomized rats. The strength of the hormone was found to be considerably lower than the reported one, however. Given in doses of up to 3.6 mg/day for 16 days, the hormone showed no secondary effect of thyrotrophic factor or growth factor. A slight probability of gonadotrophic effect may have been produced via induced hyperfunction of the adrenal cortex.

Experiment 2. Treatment of normal male rats with the corticotropic hormone.

One group of 12 animals, 33 days old, were given a dose of hormone intraperitoneally in 0.2 cc of saline daily for 30 days. Dosage: 3 animals received 2.3 mg, 3 animals 0.5 mg and 6 animals 0.1 mg per dose. Although the changes in the pituitaries were somewhat clearer after the largest doses than after the smallest, these differences within the group were not great, and, with regard to body weight and organ weights, no systematic differences at all were seen within the group. It was therefore treated as a unit.

A second group of 6 animals, 33 days old, were similarly given 0.1 mg hormone daily for 104 days.

6 littermate males served as controls for the first group, and 5 other littermate males for the second. As the controls in no respect differed from the 7 normal controls in the next experiment (No. 3), they have all been made into one control group, comprising 18 normal male rats. A further extension of the normal material as regards body weight, adrenal weight and pituitary weight was made as follows.

Since the regression line for the body weight according to age of the 18 control animals proved almost exactly to coincide with the corresponding regression line for the remaining 110 male rats from all the experiments (*one* body-weight value taken for each animal at the last age when the animal was still normal, i. e. untreated), this has extended the normal material for body weight to 128 animals. — The adrenal material could be extended in the same way, since it was found that the weight according to age of the 18 above-mentioned controls agreed very nearly with the corresponding weight of the extirpated left adrenal from normal male rats. The material has therefore been made into one (one value for each animal). The collected control material for adrenal weight covers 75 animals. — The weight according to age of the pituitaries of the 18 controls differed only by about 3 % from the corresponding weight in the 14 male rats treated solely with desoxycorticosterone acetate (DOCA) in Experiment 3. They have therefore been made into a single control group (32 animals) for pituitary weight.

The results of the hormone treatment of normal males is shown by the following curves.

As fig. 1 shows, the hormone treatment raised the adrenal weight significantly above the normal, almost equally in both treated groups. On the other hand, the histology of the adrenals was not changed.

Fig. 2 shows that the body weight was unaffected after 30 days, but had risen significantly above the normal after 104 days of treatment. Since the kidneys did not increase in weight (the mean was slightly lower in both groups of hormone-treated animals than in the littermate controls), the increase in body-weight among the animals treated for 104 days can probably not be attributed to admixture of

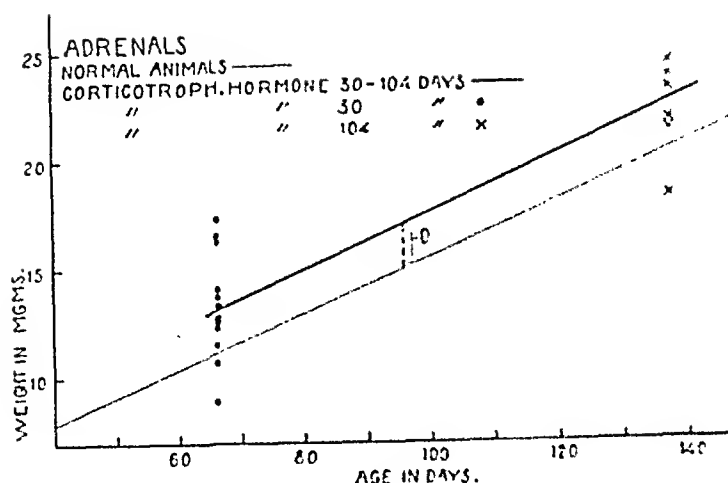


Fig. 1.

Weight according to age of adrenals of 75 normal rats, and that of 18 male rats treated with corticotrophic hormone for 30-104 days. $D = \pm 2.16 \pm 0.66$ mgms, calculated for all the hormone-treated animals.

growth factor in the hormone doses, or to raised excretion of such a factor from the pituitary, but can be assumed to be an effect of induced adrenal cortex hyperfunction: an increased fatty deposit both from corticotrophic hormone and from cortin has been shown by Reiss and co-workers (1937, 1944). — The weight of the testes, accessory sex organs, and thyroid was not affected by the hormone treatment in any group.

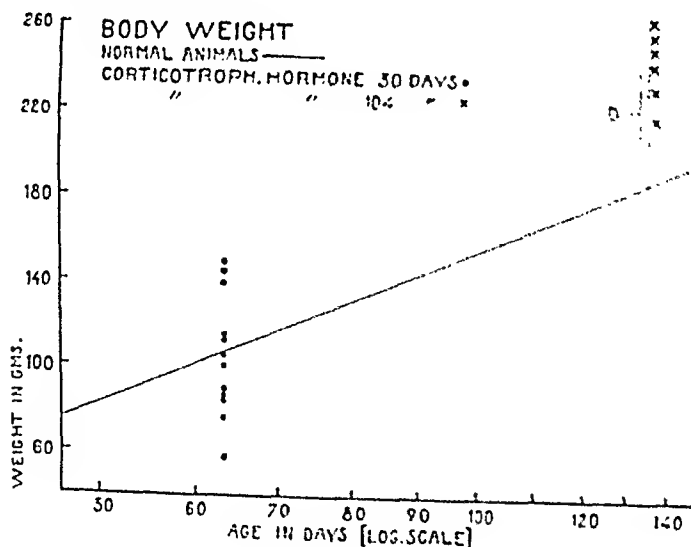


Fig. 2.

Body weight according to age of 128 normal male rats, and that of 18 male rats treated with corticotrophic hormone for 30-104 days. $D = \pm 53 \pm 7.5$ gms, calculated for animals treated for 104 days.

Table 2.

Frequency of different cell types in the anterior pituitary of male rats. Values significantly deviating from those of the 18 normal animals are printed in heavy type.

Groups of animals	Number of animals	Frequency of the different cell types in % $M \pm \epsilon(\lambda)$					
		Chromophobes, total	Acidophiles	Basophiles, total	Hypertrophic amphophiles	Pycnotic basophiles	Hyalin basoph
Animals treated with corticotroph. hormone							
for 30 days	12	48.6 \pm 0.68	36.3 \pm 0.60	15.1 \pm 0.36	0.61 \pm 0.11	1.41 \pm 0.22	0 \pm 0
for 104 days	6	43.8 \pm 0.62	43.4 \pm 0.91	12.8 \pm 0.65	0.36 \pm 0.18	1.96 \pm 0.20	0 \pm 0
Normal animals.....	18	48.5 \pm 0.42	36.7 \pm 0.40	14.8 \pm 0.28	0.63 \pm 0.07	0.27 \pm 0.06	0 \pm 0
Normal animals treated with DOCA only.....	14	48.8 \pm 0.59	36.2 \pm 0.43	14.9 \pm 0.08	0.49 \pm 0.36	0.51 \pm 0.09	0.02 \pm 0
Completely adrenalect. animals treated with DOCA							
10—18 d. after op.	13	51.3 \pm 0.67	34.5 \pm 0.65	14.1 \pm 0.58	2.90 \pm 0.24	0.86 \pm 0.10	1.26 \pm 0
24—28 d. after op.	6	52.1 \pm 0.21	34.8 \pm 0.74	13.2 \pm 0.68	2.63 \pm 0.22	0.47 \pm 0.07	2.01 \pm 0
65—86 d. after op.	10	55.3 \pm 0.67	33.0 \pm 0.57	11.6 \pm 0.61	4.64 \pm 0.17	0.61 \pm 0.06	2.13 \pm 0
Incompletely adrenalect. animals treated with DOCA	3	48.9 \pm 0.17	38.8 \pm 0.21	12.4 \pm 0.23	2.44 \pm 0.36	0.03 \pm 0.03	0.16 \pm 0

In both groups of hormone-treated animals, the weight of the pituitary proved slightly larger than in the groups of littermate controls; however, the difference between treated cases and controls was not statistically significant ($D = + 0.46 \pm 0.33$ mgms). Nor did comparison with the expanded normal material (32 controls in all) give any statistically probable difference in the weight of the pituitary ($D = + 0.15 \pm 0.28$ mgms).

The histological picture of the anterior pituitary was greatly affected by the hormone treatment, particularly the longer one; see table 2 and fig. 3.

Thus, the frequency of pycnotic basophiles was significantly raised in the group of animals treated for 30 days. The same group also showed a positive correlation between the size of the dose and the frequency of pycnotic basophiles: with a dose of 2.3 mg per day, the mean of pycnotic basophiles was 2.1 %, with a dose of 0.5 per day, it was 2.0 % and with a dose of 0.1 mg per day it was 0.7 %. — Further, after 104 days of treatment, the number of chromophobes had significantly fallen, the number of acidophiles significantly risen, and the total number of basophiles fallen (with a probability of 99:100). In this group, too, the pycnotic basophiles were most numerous (cf. figs. 3, 9 and 10).

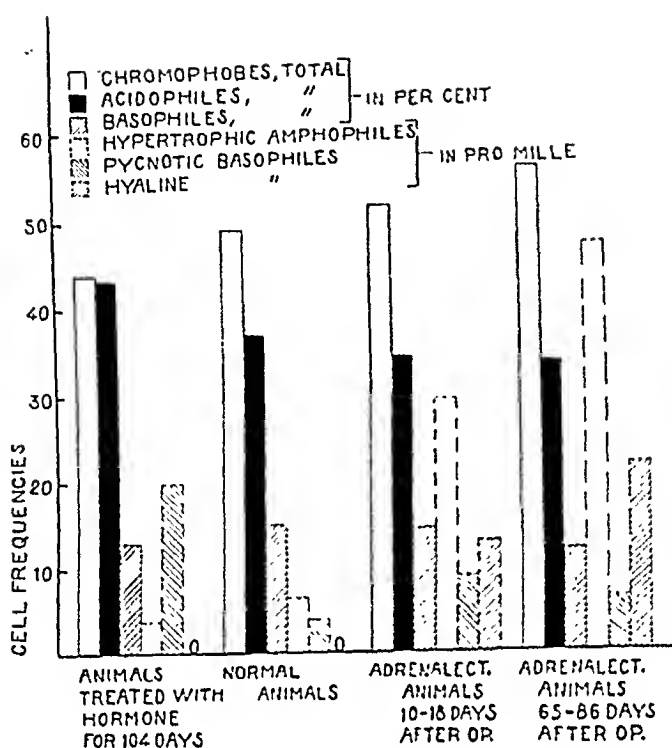


Fig. 3.

Frequencies of different cell types in the anterior pituitary of male rats treated with corticotrophic hormone, normal male rats and adrenalectomized male rats.

Summing up, we can say that, in normal male rats, Reiss's corticotrophic hormone produced a relative increase in the weight of the adrenals, and gradually a relative increase of the body weight — the latter probably due to enhanced function of the adrenal cortex. There were no signs of deranged thyrotrophic or gonadotrophic pituitary function. The anterior pituitary showed in the first place, pycnosis of basophiles, followed gradually by a lowered number of chromophobes, raised number of acidophiles, and probably a lowered number of basophiles.

The appearance of the pituitary change clearly indicates lowered basophilic activity. As no signs of disturbances of other functions of the pituitary basophiles were evinced in the experiment, it is very likely that the pituitary change implies a reduced production of corticotrophic hormone. As, moreover, it is known that hyperfunction of the adrenal cortex reduces the pituitary's excretion of corticotrophic hormone, it is likely that the hormone injections of the experiment produced the pituitary change via such adrenal hyperfunction.

As regards the inactivation of the pituitary basophiles, the results of this experiment agree well with those of Koneff's, although the

latter used another corticotrophic hormone, and far larger doses (see above). The results argue plainly against the assumption that the picture of marked basophile activity which characterises the anterior pituitary in the adrenogenital diseases is due to hyperfunction of the adrenal cortex in these diseases.

Experiment 3. Adrenalectomies.

First group: 22 male rats, 35—45 days old, were subjected to left-sided adrenalectomy, and 4—14 days later right-sided adrenalectomy. For a few days after the second operation, the animals were given by the intramuscular route 1—0.5 mg DOCA, shaken up in 0.03—0.06 cc saline; no further treatment. The animals were killed when they were practically in agone; this occurred after 10—28 days. None of the animals showed adrenal remains on dissection.

A second experiment group of 11 male rats, 38—45 days old, were subjected to left-sided adrenalectomy, followed 7—14 days later by right-sided adrenalectomy. After the second operation, the animals were given 0.50—0.25 mg of DOCA, shaken up in 0.03—0.06 cc of saline, intramuscularly every day for 23 days. During this time the body weight rose rather more than normally. For the following 13—20 days the animals were given rapidly decreasing DOCA doses until the body weight was falling steadily, which occurred at a dosage of 0.075—0.050 mg per day. The animals were kept on this dose for a further 21—38 days, after which they were killed. Many were then extremely ill, some of them dying. They had been totally adrenalectomized for 65—86 days. No animal in this group showed any adrenal remains on dissection.

A third group of 3 male rats, from the same litter as the animals in the second group, was given the same treatment as this latter group, although the body weight never fell. On dissection all 3 showed traces of adrenals, 10.6, 11.3 and 19.3 mgm in size.

The same expansion of the control material took place in experiment 3 as in experiment 2 (see above). In addition, the weights of the thyroid and of the prostate + seminal vesicles of the hormone-treated animals in experiment 2 were used in the control material, since these weights did not differ from the normal.

Results. As appears from fig. 4 there was a gradual fall in the final body weight after *complete adrenalectomy* and during the DOCA dosage, when compared with that of the normal animals. The body weight is, with a probability of 98.5:100, lower than normal in the animals 65—86 days after adrenalectomy.

The thyroid was with certainty lighter than normal only in the group kept alive 65—86 days after adrenalectomy (fig. 5), but it was already showing histological signs of incipient atrophy 25 days after the operation. That is to say, some of the follicles were enlarged, the epithelium was low in parts, and some of the colloid denser than

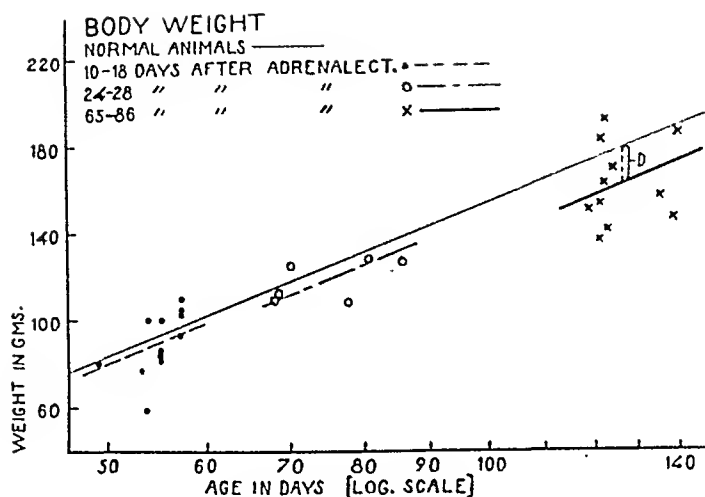


Fig. 4.

Body weight according to age of 128 normal male rats, and that of adrenalectomized males at different times from the operation. $D = -17 \pm 6.4$ gms.

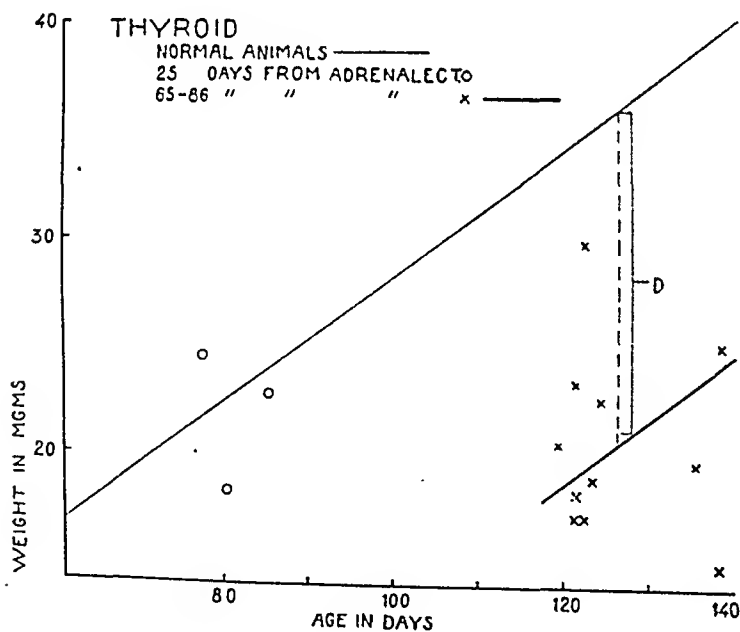


Fig. 5.

Thyroid weight according to age of 28 normal male rats (including hormone-treated animals, see text), and that of adrenalectomized males.

normally. Atrophy of the same type but far more pronounced, appeared regularly 65—86 days after the operation. — The parathyroids, on the other hand, showed no certain changes in any group. The accessory sex organs (prostate + seminal vesicles) showed definitely retarded growth even 25 days after the operation, and this was seen

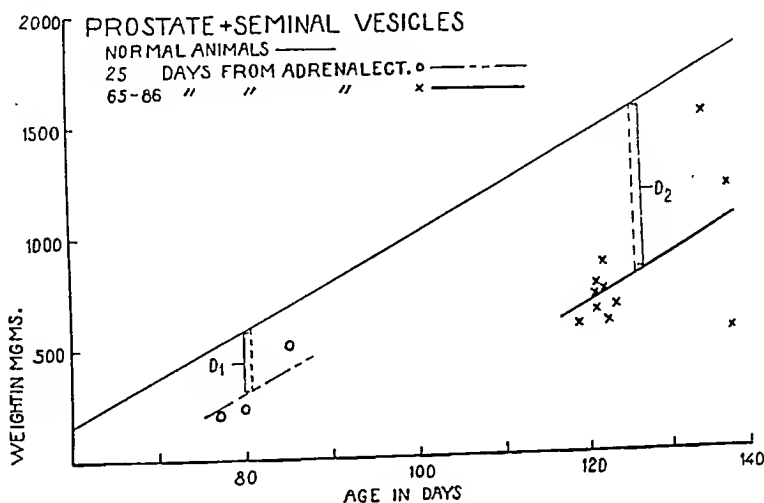


Fig. 6.

Weight of prostate + seminal vesicles according to age of 29 normal male rats (including hormone-treated animals, see text), and that of adrenalectomized males. $D_1 = -291 \pm 71$ mgms; $D_2 = -758 \pm 95$ mgms.

more clearly after 65—86 days. A histological atrophy ran parallel with the relative loss of weight; the epithelium in both organs became lower, finally resembling endothelium in parts, and the stroma of the vesicles became coarser.

25—86 days after the operation, the testes merely suggested retarded growth ($D = +65 \pm 37$ mgms); the kidneys likewise ($D = +73 \pm 43$ mgms). Neither testes nor kidneys differed histologically from the normal.

The pituitary weight (fig. 7) rose significantly above the normal 18—25 days after adrenalectomy, but in the completely adrenalectomized animals was lower than normal 65—86 days after the operation.

Table 2 and fig. 3 give the main outlines of the histological changes in the anterior pituitary at different times after complete adrenalectomy: There was a progressive increase of the number of chromophobes and a decrease in the number of chromophiles, together with an increase of hypertrophic amphophiles and hyaline basophiles. The increase of pycnotic basophiles, on the other hand, was relatively small, and possibly reached its maximum in the period when the adrenal insufficiency was most acute (10—18 days after the operation).

tion). These numerically registered changes were accompanied by a perceptible increase in the size of the basophiles; they developed enlarged nuclei and became sparsely granulated. The dividing line between such basophiles and hypertrophic amphophiles was quite indistinct as early as 24 days after the operation. Plainly, basophiles turned over into hypertrophic amphophiles.

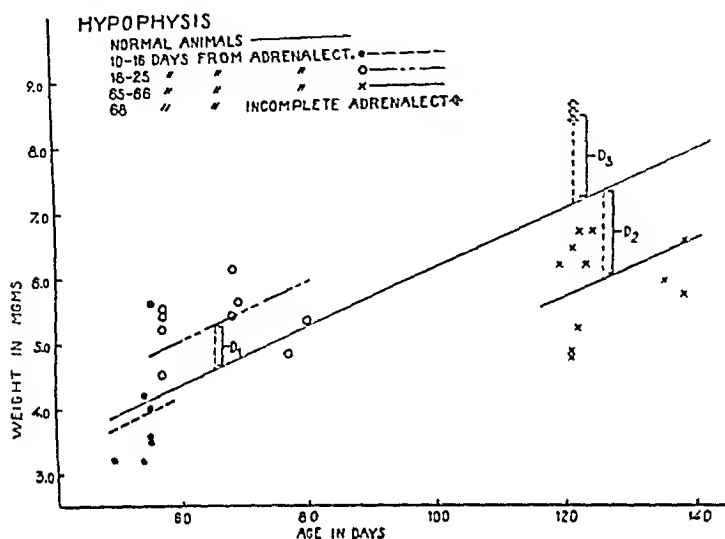


Fig. 7.

Weight of pituitary according to age of 32 normal male rats (including 14 DOCA-treated animals, see text), and that of adrenalectomized males at different times after the operation. $D_1 = +0.75 \pm 0.24$ mgms. $D_2 = -1.33 \pm 0.37$ mgms. $D_3 = +1.41 \pm 0.29$ mgms.

Most of the registered hyaline basophiles were large cells with large nuclei and hyaline scales in the protoplasm — i. e. like the pathological basophiles observed in Addison's disease. But some of these cells in the rats (particularly later than 24 days after the operation) showed real hyaline fields in the protoplasm, and were strikingly like Crooke's hyaline basophiles in Cushing's disease — if indeed, they were not definitely identical (figs. 11 and 12).

On the other hand, the acidophiles, though fewer than normal, showed no cytological changes.

In the *incompletely adrenalectomized* animals, the body weight 67 days after the operations was found to be higher than normal ($D = +35 \pm 12$ gms; P about 0.004), the kidneys possibly heavier than normal ($D = +232 \pm 96$ mgms), the thyroid, on the other hand, certainly lighter than normal ($D = -12 \pm 1.6$ mgms) but the testes ($D = +11 \pm 36$ mgms) and the accessory sex organs ($D = +14 \pm 36$ mgms) normal in weight. The pituitary was heavier than normal (cf. fig. 7); histologically it showed only an increased number of acidophiles and hypertrophic amphophiles and a lowered number of basophiles (cf. table 2).

Summing up, we can say that, after about a month, complete (but *not* incomplete) adrenalectomy of young male rats, combined with the minimal DOCA dosage, produced progressive hypotrophy of the thyroid gland, the prostate gland, and the seminal vesicles, together with gradual loss of weight. The parathyroids were not changed histologically, and the testes and kidneys showed no definite effect, either as regards weight or histology.

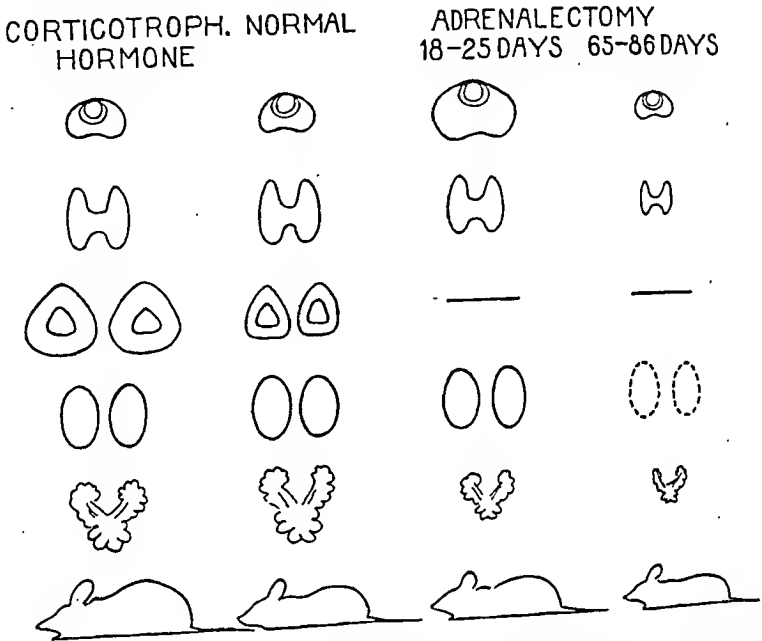


Fig. 8.

Diagram of weight of endocrine organs and body weight of male rats treated with corticotrophic hormone, normal rats, and adrenalectomized rats.

The results agree in the main with those of Grollman and Firor (1935) which authors obtained retardation of body growth and hypotrophy of the thyroid and sex organs in chronic adrenal insufficiency in rats. They also showed that, after a time, adrenal cortex extract was not able to restore these changes, but that pituitary extract was.

It is therefore clear that the hypotrophy of the thyroid and the sex organs after adrenalectomy depends, at all events in part, on reduced excretion of thyrotrophic and gonadotrophic hormones from the anterior pituitary. It is also known that the basophile pituitary cells are involved in the production of the very hormones mentioned, as also in the formation of the corticotrophic hormone.

In spite of this, the present author found adrenalectomy to be followed by distinct and gradually increasing histological signs of raised basophilic activity of the anterior pituitary. That is to say, the operation caused a shift of the pituitary hormone production, in all probability to increased production of corticotrophic hormone. This

conception tallies with the earlier findings of raised concentration of corticotrophic hormone in the anterior pituitary and the blood after adrenalectomy (see above).

Although the pituitary change in the adrenalectomized rats is like that in Addison's disease, there is a difference in that the hypertrophy of the basophiles is far in excess of regressive changes. This may be because the experiments were too acute: the weight curve of the pituitary after adrenalectomy, first rising and then falling in relation to the normal weight, may indicate a gradual regression of the temporarily raised function.

On the other hand, it is plain that the pituitary change in the adrenalectomized animals very greatly resembles that in the adrenogenital diseases. The results strongly suggest that the pituitary change in these diseases involved an enhanced corticotrophic function, producing hyperfunction of the adrenal cortex as a secondary effect.

Summary

It has previously been shown that the adrenogenital diseases in man are all characterised by fundamentally the same histological change in the anterior pituitary. The change, whose appearance indicates a strong basophilic activity, sets in regardless of whether the adrenal cortex in the individual case is the seat of adenoma, cancer, or merely hyperplasia, but it is most perceptible when the adrenal growth is greatest.

The question therefore is whether this pituitary change is the result of hyperfunction of the adrenal cortex, or whether it implies a raised corticotrophic function, which produces hyperfunction of the cortex in the adrenogenital diseases.

Certain earlier observations suggest that the latter alternative is the correct one. For example, Addison's disease has been observed to show pituitary changes indicating raised basophilic activity similar to that in the adrenogenital diseases. A new case of this kind is reported.

The question has been illustrated experimentally by the following three tests, carried out on a total of 147 white rats (128 males + 19 females, including controls).

1. Control tests of Reiss's corticotrophic hormone were made on 36 hypophysectomized animals. The hormone had a sure corticotrophic (antisudanophobic) effect, but no secondary effect of thyrotrophic factor or growth factor. A slight likelihood of gonadotrophic effect may have arisen through raised adrenal cortex function.

2. The same corticotrophic hormone was given to 18 normal male rats for 30—104 days. The adrenals became heavier. The body weight gradually rose, probably due to enhanced function of the adrenal cortex. The anterior pituitary showed, primarily, pycnosis of the basophiles; gradually, there was also a fall in the number of chromophobes, a rise in the acidophiles, and probably a fall in the basophiles.

No hyaline basophiles appeared, and the number of hypertrophic amphophiles did not increase. The change indicates reduced basophilic activity, probably corresponding to a lowered corticotrophic function.

3. 33 male rats underwent complete adrenalectomy, and were kept alive 10—86 days with an inadequate, decreasing dose of desoxycorticosterone acetate. After about a month, successive hypotrophy of the thyroid, the prostate, and the seminal vesicles set in; with time, there was also a relative fall in body weight. The weight of the pituitary began by rising, and then fell, in relation to the normal weight. The anterior pituitary showed clear and increasing signs of enhanced basophilic activity; the basophiles became larger, passed over into hypertrophic amphophiles, and hyaline basophiles appeared, some of them very like Crooke's hyaline basophiles in the adrenogenital diseases. The total number of chromophiles fell, and the number of chromophobes rose. In all probability, the pituitary change indicates a raised production of corticotrophic hormone.

The results argue strongly against the assumption that the pituitary change in the adrenogenital diseases are a result of hyperfunction of the adrenal cortex. They strongly suggest that the same pituitary change corresponds to a raised production of corticotrophic hormone, which produces hyperfunction of the adrenal cortex.

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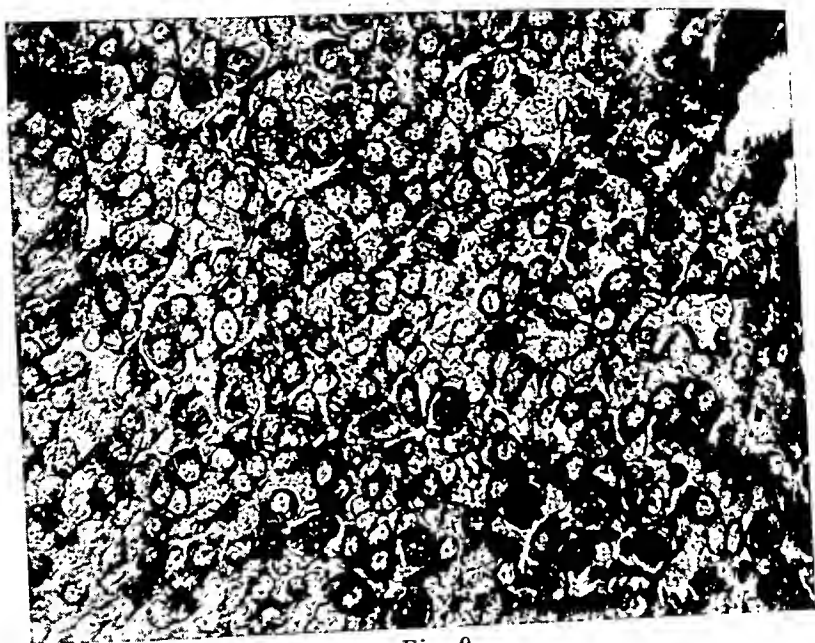


Fig. 9.

Anterior pituitary of 137-day-old normal male rat. Stained according to Mellgren. — $\times 550$.

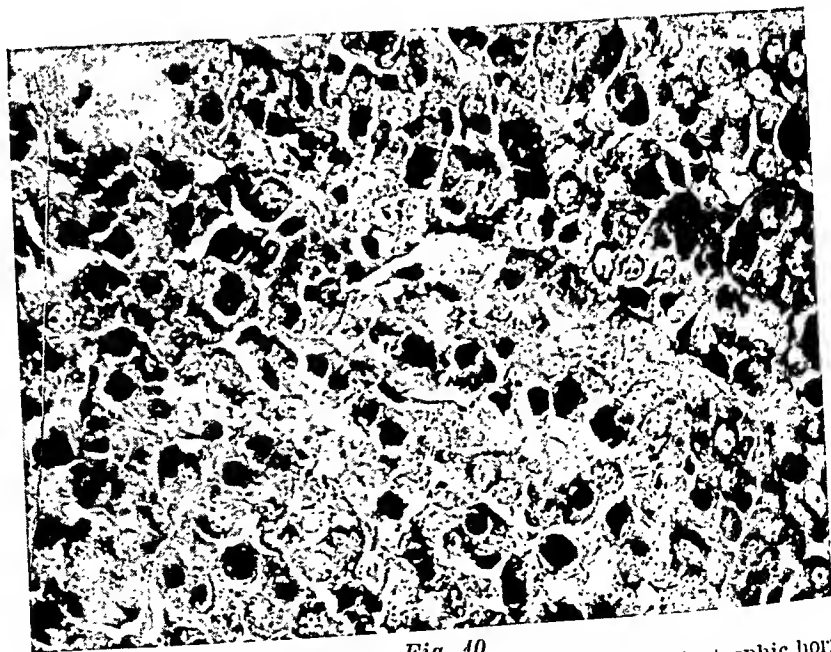
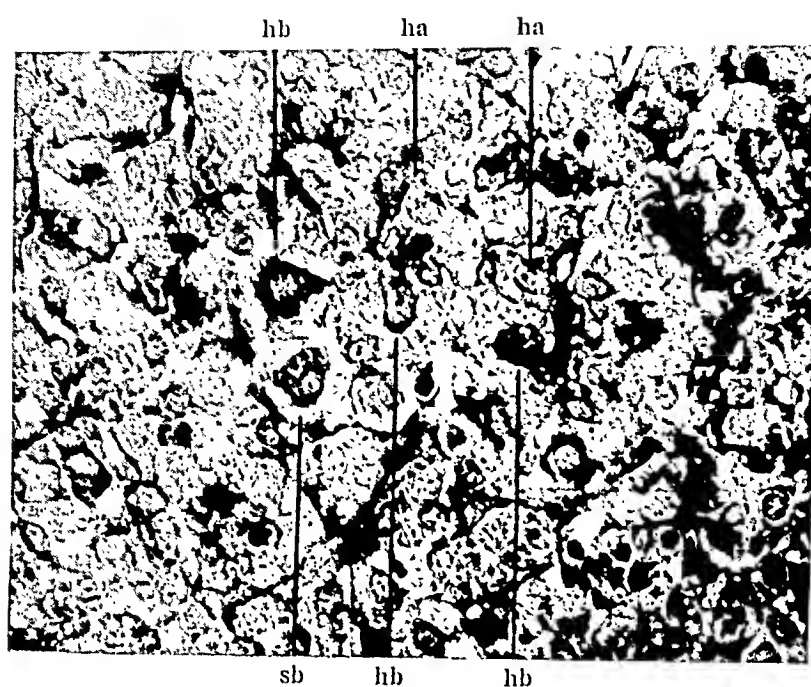


Fig. 10.

Anterior pituitary of 137-day-old male rat treated with corticotrophic hormone for 104 days. Note numerous pycnotic basophiles. Stained according to Mellgren. — $\times 550$.

*Fig. 11.*

Anterior pituitary of 138-day-old male rat 70 days after complete adrenalectomy. Hypertrophic amphophiles (ha), sparsely granulated basophiles (sb) and hyaline basophiles (hb). — Stained according to Mellgren. — $\times 550$.

*Fig. 12.*

Central portion of fig. 11. — $\times 1700$.

CAPSULAR ANTIGENS IN SOME NON-HAEMOLYTIC STREPTOCOCCI BELONGING TO THE LANCEFIELD GROUP D.

By Knud Skadhauge.

(Received for publication September 27th 1947).

There is very little information in the literature about capsules in non-haemolytic streptococci. The *Streptococcus bovis*, described by Orla-Jensen, 1919,¹) is said by this author to be able to form capsules in milk. Lewcoviez²) and later Schmitz³) claim to have seen capsules in enterococci, but it is not proven that the bacteria investigated by these authors were enterococci as the term is understood to-day. Also Dible,⁴) Bagger⁵) and in later years Sherman and his collaborators have been unable to demonstrate capsules in these organisms. In these papers the investigations on capsules have been carried out in all cases by non-serological methods.

In 1944⁶) and 1945⁷) Mørch described two strains of *Streptococcus salivarius* giving capsular swelling a. m. Neufeld not only with homologous serum but also with some antipneumococcal sera. In one of these cases Mørch succeeded in staining the capsules with the technique of Muir, although it was not possible to demonstrate capsules in India-ink preparations; while in the other case capsules were clearly demonstrated by means of India-ink, but no success was obtained by Muir's method.

Authors own investigations.

During systematic investigations on the serology of enterococci it was found that capsular swelling was produced by addition of homologous serum in a number of strains. The present paper contains a detailed account of the serological properties of these strains. It is thought that this may be of interest since capsules in enterococci as mentioned above have not hitherto been found, and especially since no information seems to have been published about capsular antigens in enterococci and related bacteria.

The term »enterococci« has been defined somewhat variously by different workers, but it is possible to enumerate certain cultural and physical-chemical properties which seem to be especially characteristic.

According to *Sherman* and collaborators⁸⁾ the bacteria of the enterococcus group are distinguishable from the other non-haemolytic streptococci by their:

- 1) ability to grow at both 10° C and 45° C,
- 2) ability to grow in the presence of 6.5 per cent NaCl and at pH 9.6.

Other characteristics are the resistance of enterococci, to:

- 3) Heat (resisting 60° for 30 minutes),
- 4) methylene blue (growth in the presence of 0.1 per cent methylene blue) and
- 5) their powerful reducing action (reduce litmus or methylene blue in milk *before* coagulation).

On the basis of their cultural features *Sherman*⁹⁾ sets up the following types within the enterococcus group:

Streptococcus fecalis and

Streptococcus liquefaciens, both of which are non-haemolytic and mannitol-fermenting. *Str. liquefaciens* liquefies gelatin and may be regarded as a gelatin-liquefying variant of *Str. fecalis*.

Streptococcus zymogenes and

Streptococcus durans, both of which are beta-haemolytic. *Str. zymogenes* liquefies gelatin. In contrast to the other three types, *Str. durans* has a weak reducing action only and usually does not ferment mannitol.

In addition to these four types there is a beta-haemolytic, mannitol-fermenting, non-gelatin-liquefying variant of *Str. fecalis*.

It is of particular significance in the diagnosis of the bacteria of the enterococcus group that *Sherman*¹⁰⁾ as well as *Graham & Bartley*¹¹⁾ have been able to show that strains of all these four types belong to Lancefield's group D. Thus in recent years there is an inclination to regard the term enterococcus as being synonymous with group D *Streptococcus*.

The strains examined.

The material comprises 12 strains (from a total of about 300 enterococcus strains). Eight of the strains were isolated from infected appendices removed by appendicectomy (Nos. 7, 11, 15, 19, 20, 21, 22 and 36), and the other four were cultivated from human feces; the specimens had been submitted for examination for pathogenic intestinal bacteria, but these were not found present.

Cultural features.

In all cases the organisms were cocci which grew diffusely in fluid medium, and which were Gram-positive, oval to lanceolate diplococci, only exceptionally arranged in short chains.

On agar plates containing 5 per cent horse blood they grew in the form of pin-head sized whitish colonies with a smooth surface and soft consistency, surrounded by a very narrow border of alpha-haemolysis.

All the strains were capable of growing in broth with 6.5 per cent NaCl and at pH 9.6 as well as in skimmed milk with 0.1 per cent methylene blue.

None of the strains liquefied gelatin.

All the strains grew in broth at 10° C and at 45° C, and nine of them were able to grow at 50° C also.

Heat-resistance tests showed that all the strains survived heating at 61° C for 30 minutes. Strains Nos. 7, 22, 157 and 244 resisted heating at 63° C, while Nos. 11, 15, 19, 20 and 21 tolerated heating at 65° C for 30 minutes.

The heat-resistance tests were made in the following manner: about 2 ml of a 20-hour broth culture were placed in a short test-tube which was closed with a sterile rubber stopper, whereafter it was lowered into a waterbath of the desired temperature. After exactly 30 minutes the tubes were lifted out, cooled briefly under the tap and then incubated at 37° C for about 20 hours. The incubated tubes were then seeded in broth and on to blood-agar plates.

The reducing power of the strains was examined by seeding one Pasteurpipette drop of a 20-hour culture into skimmed milk (about 8 ml) containing 0.02 per cent methylene blue. The reaction was read off after incubating at 37° C for: 2, 4, 6, 8, 12, 24 and 48 hours respectively. The degree of reduction was expressed as: (+), +, ++ and +++, and the moment of coagulation was marked (C).

The results of these tests are shown in fig. 1:

Fig. 1.

Strain No.	Degree of reduction after number of hours						
	2	4	6	8	12	24	48
7	0	0	0	0	0	0	0
11	0	0	0	0	(+)	++	++
15	0	0	0	0	(+)	+++ C	
19	0	0	0	0	0	0	+++ C
20	0	0	0	0	0	0	++
21	0	0	0	0	0	0	++
22	0	0	(+)	(+)	+	++	+++ C
36	0	0	0	(+)	++	+++ C	
155	0	0	0	0	0	++	+++ C
157	0	0	+	++	+++	+++ C	
158	0	0	0	0	0	0	0
244	0	0	+	++	+++ C		

It was found that a considerable number of typical enterococcus strains tested by this technique showed strong reduction within the first 6 hours, and therefore it was considered justifiable to regard the present strains as weak- or non-reducing.

For purposes of comparison the result of reduction tests on 10 arbitrarily selected typical enterococcus strains are appended. (fig. 2).

Fig. 2.

Strain No.	Degree of reduction after number of hours		
	4	6	12
180	++	+++	+++
255	++	+++	+++
363	++	+++	+++
438	++	+++	+++
465	++	+++	+++
496	++	+++ C	
503	++	+++	+++ C
537	++	+++ C	
539	++	+++	+++
541	++	+++	+++

The strains were tested for their ability to ferment arabinose, lactose raffinose, inulin, glycerol, mannitol, sorbitol, salicin and aesculin.

Except as regards their action on glycerol, all the strains behaved in the same manner, all fermenting arabinose, lactose, mannitol, salicin and aesculin within 24 hours. One strain (No. 11) fermented glycerol within 24 hours, whereas with the others the reaction was not acid until the third day.

None of the strains fermented raffinose, inulin or sorbitol.

Thus the strains investigated differed from the classical picture of the bacteria of the enterococcus group by their inability, or only weakly manifested ability, to reduce methylene blue in skimmed milk, and by their inability to ferment sorbitol.

Serology.

Precipitation tests were made with all the strains, using sera of Lancefields groups A, B, C, D, E, F, G, H and K. The only positive reaction was with a group D serum, which precipitated all the strains. The group D serum employed was prepared by immunizing with a strain of *Str. durans* by a method suggested by *Shattock*, and *Mattick*.¹²⁾ This serum had been kindly placed at my disposal by Dr. Shattock, The Institute for Research in Dairying, Shinfield nr. Reading, England.

Using all 12 strains rabbit-immune sera were prepared, employing formol-killed culture as the antigen.

Fig. 3.
The cultural features of the strains examined.

Strain	Hemolysis	Gelatin Liquefaction	Strong reduction	Milk curdled	Growth at				Growth in presence of			Survival 60 ° C., 30 minutes	Acid produced from									
					10 ° C	45 ° C	50 ° C	40 per cent bile agar	6.5 per cent NaCl	pH 9.6	0.1 per cent methyleneblue		Arabinose	Lactose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	Esculin	
7	—	—	—	—	+	+	—	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
11	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
15	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
19	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
20	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
21	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
22	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
36	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
155	—	—	—	+	+	+	—	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
157	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
158	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+
244	—	—	—	+	+	+	—	+	+	+	+	+	+	+	+	—	—	+	+	—	+	+

The results of cross-agglutination in a waterbath (50°) read after 20 hours, will be seen from fig. 4.

Fig. 4.

Formol-killed cultures	Unabsorbed sera										
	7	11	15	19	20	21	22	155	157	158	244
7	320	0	0	0	0	0	0	0	0	0	0
11	0	320	0	0	0	0	0	0	0	0	9
15	0	0	320	0	0	0	0	0	0	0	0
19	0	0	0	320	0	0	0	0	0	0	0
20	0	0	0	0	640	0	0	0	0	0	0
21	0	0	0	0	0	320	0	0	0	0	0
22	0	0	0	0	0	0	80	0	0	0	0
155	0	0	0	0	0	0	0	320	0	0	0
157	0	0	0	0	0	0	0	0	320	0	0
158	0	0	0	0	0	0	0	0	0	320	0
244	0	0	0	0	0	0	0	0	0	0	640

Obs! The strain No. 36 was found to be identical with strain No. 15.

It was remarkable that the homologous titre for these strains was so relatively low, seeing that agglutination tests with a large number of typical enterococcus strains had shown that it was not difficult to obtain sera with a titre of 1280 up to 5120. The possibility was considered that these strains possessed an antigen which was localized to

the surface of the bacteria, perhaps a capsular antigen, which of itself gave a low titre and at the same time »covered« more deeply seated antigens. For the purpose of discovering the presence of such a capsular antigen, all the strains were tested by the Neufeld method, a drop of a culture on a slide being mixed with a drop of undiluted homologous serum. Microscopic examination then revealed that when homologous serum was added all 12 strains gave a swelling reaction, but it was impossible in the unstained slide to distinguish definitely between »capsule« and cell body. The intensity of the reaction varied somewhat in the different strains, but it was clearly seen in every case. In the most marked cases the volume of the bacterium increased to two or three times the original volume. On testing with a series of serum dilutions it was found that the titre of this swelling reaction was fairly uniform for all the strains, ranging from 16 to 64. The reaction was quite type-specific, appearing only after the addition of homologous serum.

By staining the cultures with the method of Muir and by making India-ink preparations without adding serum no demonstration of capsules was obtained. However, it seems doubtful how much weight should be attached to a negative result from staining methods and India-ink preparations. *Morch's* experiments, in which these methods gave varying results, despite the fact that capsules were distinctly observed when serum was added, seem to argue that these methods are rather uncertain.

In the demonstration of capsules in a *Coli* strain *Morch & Knipschildt*¹³⁾ stained the culture with a saturated solution of methylene blue *simultaneously* with the addition of the serum. The result was a handsome contrast between the stained cell body and the unstained, clear capsule.

Using the same technique on the strains investigated it was possible to distinguish between cell body and capsule, but the picture was scarcely as clear as in *Morch & Knipschildt's* case.

However, when using a concentrated solution of fuchsin and the same technique distinct capsules were demonstrated in all 12 strains. The contrast between the deeply red stained cell bodies and the clear, unstained capsules became very sharp after the fuchsin had acted for about ten minutes.

Accordingly it seemed justifiable to regard the above mentioned swelling as a capsular swelling reaction.

In order to decide whether the age of the culture played any part in this reaction the Neufeld-test was carried out with cultures which had been incubated for 2, 4, 6, 12 and 20 hours, and finally with cultures incubated at 37° C for about 20 hours and then left at room temperature for three weeks. In all cases the capsular swelling reaction was distinctly marked, indicating that the age of the culture is of no special significance.

Nor did the medium used for the cultures seem to play any role, provided one used a medium in which the bacteria grew well. The reaction was tested with cultures from trypsin broth, serum broth and ordinary filtered broth with 1 per cent glucose added, and was found to be equally pronounced in all cases.

In order to decide whether trypsin treatment would destroy the antigen necessary to the reaction, cultures were employed which had been treated for 2 and 24 hours with trypsin. There was distinct capsular swelling in both cases. Thus trypsin treatment does not seem to destroy the capsular antigen.

In analogy with the serological procedure for the bacteria of the Coli group, in which the O-inagglutinability conditioned by capsular antigens can be abolished by subjecting the culture to heat, all cultures were autoclaved at 127° C for two hours and then used as antigen in the Neufeld test as well as in the agglutination test. It was then found that after autoclaving capsular swelling could still be demonstrated in strains Nos. 7, 19, 20, 21, 22, 157, whereas it was lost in the other strains. It would thus seem that as regards some strains autoclaving destroys the antigen responsible for the capsular swelling reaction.

The results of cross-agglutination tests in the waterbath, employing autoclaved cultures as antigen, will be seen from fig. 5:

Fig. 5.

Autoclaved cultures	Unabsorbed sera										
	7	11	15	19	20	21	22	155	157	158	244
7	320	0	0	0	0	0	0	0	0	0	0
11	0	<u>5120</u>	0	0	0	0	0	0	0	0	20
15	0	0	<u>5120</u>	0	0	0	0	0	0	0	160
19	0	0	0	320	0	0	0	0	0	0	0
20	0	0	0	0	640	0	0	0	0	0	0
21	0	0	0	0	0	640	0	0	0	0	0
22	0	0	0	0	0	0	160	0	0	0	0
155	0	0	0	0	0	0	0	<u>5120</u>	0	0	0
157	0	0	0	0	0	0	0	0	320	0	0
158	0	0	0	0	0	0	0	0	0	<u>5120</u>	0
244	0	20	160	0	0	0	0	0	0	0	<u>5120</u>

It will be observed that for strains Nos. 11, 15, 155, 158 and 244, the homologous titre was higher than that with formol-killed cultures. At the same time there now appeared a few small overlapping reactions. The titre increase was observed in the same strains which autoclaving had deprived of their ability to give a capsular swelling reaction. This suggests that antigens, uncovered by the destruction of the capsular antigen, had now become active.

By absorbing all the sera with homologous autoclaved cultures it

was hoped to find whether antibody would be left capable of causing the capsular swelling reaction with formol-killed culture; or, in other words, whether the capsular antibody could be isolated in the manner described. These absorption tests showed that sera Nos. 7, 19, 20, 21, 22 and 157 were completely emptied by absorption with the autoclaved homologous culture. With these absorbed sera no reaction could be observed in waterbath-agglutination tests or by the Neufeld method with formol-killed cultures as antigen. Sera Nos. 11, 15, 155, 158 and 244 absorbed with autoclaved homologous cultures gave a distinct capsular swelling reaction with formol-killed cultures. The presence of capsular antibody was not demonstrated by waterbath-agglutination, however. It may be that the absence of a reaction to waterbath-agglutination was due to the agglutination titre for the capsular antibody being below the limit of the serum dilutions employed (1:20).

Discussion.

Culturally the strains examined in the present paper are closely related to the enterococci, being able to grow at both 10° C and 45° C as well as in fluid medium containing 6,5 per cent NaCl and at pH 9,6. They were likewise resistant to heat and to methylene blue in a concentration of 0,1 per cent. As regards fermentation, they differ from the classical description of the enterococci by their inability to ferment sorbitol. It is remarkable that the strains reduce methylene blue in milk weakly or not at all. *Sherman, Mauer & Stark*,¹⁴⁾ however, have observed strains of *Str. fecalis* which behaved similarly, and they also state that after prolonged cultivation in the laboratory other strains of *Str. fecalis* are liable to lose this power.

It should also be pointed out that when cultivated on blood-agar all the strains exhibited alpha-haemolysis.

As mentioned above, all the strains reacted when precipitated against a group D serum. It is debatable whether this reaction justifies the classification of these strains as enterococci. In precipitation tests with more than a hundred strains of *Str. bovis*, *Sherman*¹⁵⁾ found that about 50 per cent of them gave a positive reaction against a group D serum. In this connection it may be added that *Grumbach*¹⁶⁾ found that his enterococcus strains were strictly type-specific, using precipitation tests.

The question of the justification of regarding the enterococci and the group D streptococci as identical concepts, as also the question of the degree of specificity of a positive reaction with a group D serum, can hardly be said to have been decided at present.

The present strains differ quite distinctly from pneumococci by their resistance to bile, and can be distinguished from the group of »Viridans- streptococci« and the *Str. lactis* group by their ability to grow at both 10° C and 45° C, in media containing 6,5 per cent NaCl and in media with a pH of 9,6.

Nevertheless, although culturally these strains seem to be very closely related to the bacteria of the enterococcus group and all gave a positive precipitation test with a group D serum, it will for the present be advisable to leave open the question of classifying them as enterococci having regard to the special serological features demonstrated above.

From these investigations it seems evident that the strains in question are characterised by the possession of a type-specific antigen which produces capsular swelling when tested against homologous serum on a slide.

By means of staining with concentrated fuchsin solution and simultaneously adding homologous serum it was possible to demonstrate a distinct capsule, thus justifying regarding the antigen demonstrated as a capsular antigen.

This capsular antigen seems to be fairly resistant. It remains unchanged regardless of the age of the culture, cannot be destroyed even by prolonged trypsin treatment, and is only occasionally destroyed by autoclaving the culture at 127° C for two hours.

From agglutination tests in the waterbath, using formol-killed or autoclaved cultures as antigen, it would seem that the change in titre observed in some of the strains after autoclaving may be due to more deeply seated antigens, the reactions of which are masked by the capsular antigen when using formol-killed culture as antigen.

Summary.

The author describes 12 non-haemolytic strains of streptococci which culturally and by precipitation reactions with a group D serum are closely related to the bacteria of the enterococcus group.

Serologically these strains are characterised by possession of a capsular antigen which produces distinct capsular swelling on addition of homologous serum.

The capsule can also be demonstrated by staining with concentrated fuchsin solution and simultaneous addition of homologous serum.

There would thus seem to be a capsular antigen not previously described.

This antigen is completely resistant even to prolonged trypsin treatment and is relatively heat-resistant.

Waterbath-agglutination, using formol-killed or autoclaved cultures as antigen, gives results which seem to indicate that this capsular antigen is capable of masking the reaction of more deeply seated antigens.

By means of absorption experiments it was possible in some instances to produce sera containing the antibody responsible for the capsular swelling reaction only.

Resumé.

Description de 12 souches de Streptocoques non hémolytiques qui par leurs caractères et leurs précipitation avec un sérum du groupe D de Lancefield se rapprochent des Entérocoques.

Sérologiquement ces souches sont caractérisées par le fait que en présence d'un antisérum homologue il se produit un gonflement de capsules très net; l'adjonction simultanée d'une solution de fuchsine concentrée montre un contraste frappant entre les corps microbiens fortement colorés et leurs capsules incolores.

Il est possible de mettre en évidence un antigène capsulaire qui ne semble pas avoir été décrit jusqu'à présent.

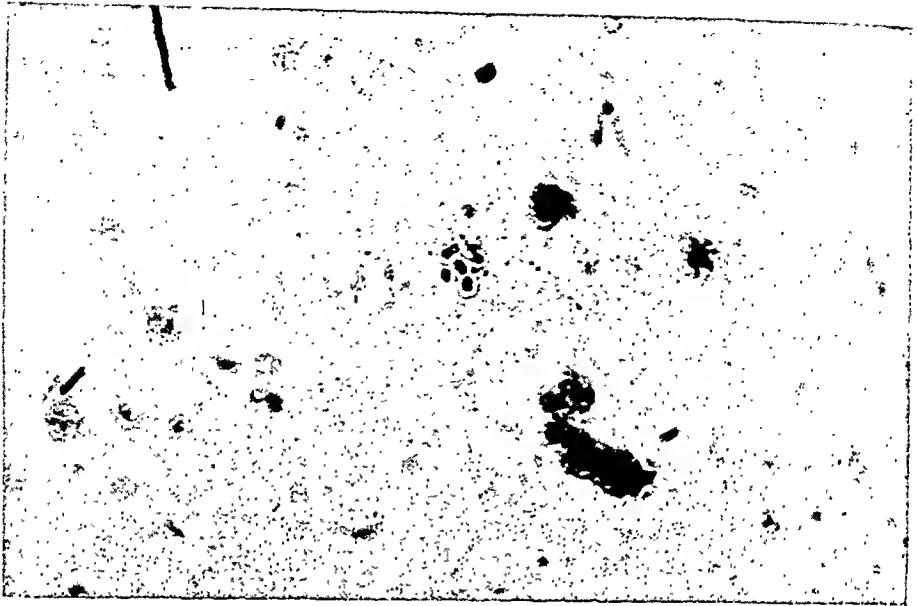
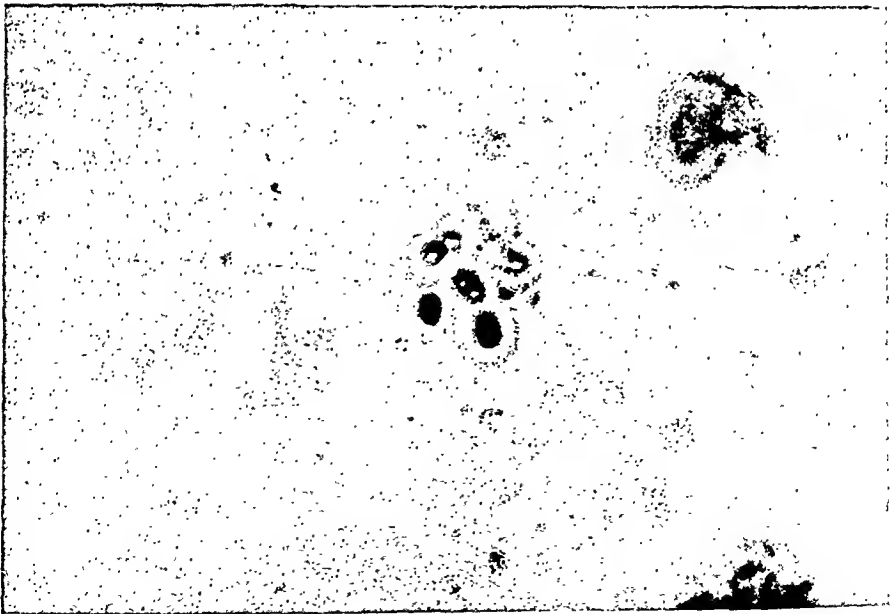
Cet antigène résiste à un traitement prolongé par la trypsine. Il est relativement thermorésistant. En effet, après deux heures d'autoclave on constate que cet antigène capsulaire est détruit dans 50 % des souches seulement.

L'agglutination au bain-marie à 50° C, pratiquée sur des germes tués soit par le formol, soit à l'autoclave aurait montré que cet antigène capsulaire semble «couvrir» d'autres antigènes.

Des épreuves d'adsorption ont permis dans certains cas d'obtenir des sérums contenant uniquement les anticorps capables de provoquer un gonflement de capsules.

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*Fig. 6.**Fig. 7.*

Demonstration of capsules by staining with fuchsin and simultaneous addition of homologous serum.

IMMUNIZATION OF ADULTS AGAINST DIPHTHERIA WITH PARTICULAR REFERENCE TO DOSAGE AND REACTIONS

By I. Scheibel, S. Tulinius, G. Rasch, K. Bojlén and Chr. Borg Petersen.

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In Denmark, as everywhere in the world, diphtheria was formerly a disease which mainly attacked children. The ratio of morbidity between the number of adults and children was fairly constantly 1:3, and the case fatality rate fluctuated between 3 and 8 %, usually being highest in years when the fewest cases of diphtheria were notified.

Matters continued in the same way in the first years of World War II, but in 1942 a change began to appear, the ratio for this year being 1:1.3 in both town and country. In 1943 it increased to 1.8:1, in 1944 to 2.6:1 and in 1945 to 2.8:1.

Before the war, practically all diphtheria was caused by diphtheria bacilli of the types *intermedius* and *mitis*, only very few cases, caused by infection from abroad, being due to the *gravis* type. The latter came to this country with the German occupation troops and a very considerable percentage of the diphtheria occurring in the later half of the war was caused by it. The fatality rate rose at the same time. In 1945 the total diphtheria fatality rate was 10.9 %, and children under 15 years had a still higher figure, 17 % (Ammundsen et al. 1946).

Table I shows the number of diphtheria cases distributed over Copenhagen, provincial towns and rural areas as well as the number of deaths from the disease and the case fatality rates, all for the years 1938 to 1945 inclusive. It may be added that there was much less diphtheria in 1946, the total number of cases being 987.

In order to meet the increased risk of epidemics, a vigorous campaign was started in the autumn of 1941 for the immunization of children, and a little later the propaganda was extended to adults. Since then, at least 90 % of the children between the ages of 1 and 15 have been immunized. A considerable number of grown-ups have

Table 1.
Diphtheria Cases and Deaths 1938—1945.

	Copenhagen			Other Towns			Rural Areas			Whole Country		
	0-14 years	> 15 years	Total	0-14 years	> 15 years	Total	0-14 years	> 15 years	Total	0-14 years	> 15 years	Total
1938 No. Died Fatality rate	240	60	300 30 10%	197	82	279 17 6.1%	205	86	291 21 7.2%	642 58 9%	228 10 4.4%	870 68 7.8%
1939 No. Died Fatality rate	316	67	383 14 3.7%	267	89	356 13 3.7%	240	109	349 25 7.2%	823 45 5.5%	265 7 2.7%	1088 52 4.8%
1940 No. Died Fatality rate	180	54	234 5 2.1%	160	105	265 17 6.4%	242	119	361 22 6.1%	582 39 6.7%	278 5 1.8%	860 44 5.1%
1941 No. Died Fatality rate	94	36	130 4 3.1%	401	163	564 11 1.9%	144	79	223 8 3.6%	639 17 2.7%	278 6 2.2%	917 23 2.5%
1942 No. Died Fatality rate	26	20	46 1 2.2%	581	357	938 40 4.3%	391	286	677 49 7.2%	988 66 6.6%	663 24 3.6%	1661 90 5.4%
1943 No. Died Fatality rate	91	126	217 18 8.3%	547	915	1462 83 5.7%	298	553	851 75 8.8%	936 98 10.5%	1594 78 4.9%	2530 176 7.0%
1944 No. Died Fatality rate	271	606	877 76 8.7%	344	1027	1371 83 6.1%	293	812	1105 96 8.7%	908 95 10.5%	2445 160 6.5%	3353 255 7.7%
1945 No. Died Fatality rate	233	649	882 77 8.7%	238	719	957 93 9.7%	239	599	838 122 14.5%	710 121 17.0%	1967 171 8.7%	2677 292 10.9%

also been immunized; there are no actual statistics, but the total may be estimated at 35 to 40 %.

The change in the ratio of the morbidity in adults to that in children is undoubtedly connected with the very widespread immunization of children and the much lower immunization figures for adults. This explanation is further supported by the fact that almost all those who contracted diphtheria during the past few years either were not immunized or had not completed the immunization (Bojén, 1943; Ammundsen et al. 1946).

Most investigations made previously on the subject of diphtheria immunization have naturally been confined to children, and therefore when adults became involved there were still a number of questions requiring elucidation. At an early stage it was noticed that adults often suffer from severe reactions during diphtheria immunization, even when the prophylactics are of a highly purified nature. For this reason, the precaution was taken to inject adults with a prophylactic only half as strong as that used for children. But notwithstanding this precaution, unpleasant complications were not avoided, it being not uncommon to have pyrexia, severe malaise and confinement to bed for several days after the inoculation. For persons who for various reasons might be assumed to be hypersensitive, or in cases where it was especially desirable to avoid a severe reaction if possible, we injected four doses of 0.2 ml. instead of the usual two doses of 1 ml., according to the common assumption that several small doses administered over a certain period produce the same or a greater effect than one large dose (Park & Schroder, 1932; Povitzky, 1932; Kern et al. 1935). This procedure for hypersensitive adults is in general use all over the world, though to our knowledge literature does not contain decisive evidence of its efficacy. Consequently, we wished to determine: 1) whether, in respect of the production of antitoxin, the »small-dose method« employed by us in certain cases is the equal of the usual routine method, 2) whether this procedure could be simplified as suggested by J. Ipsen, i. e. the number of injections being reduced from 4 to 3, 3) whether the dose (1 ml.) so far employed for mass immunization could be halved, and 4) whether the frequency and degree of the complications decreased with the dosage employed.

An investigation of this kind is only feasible if a sufficient number of volunteers are willing to give the necessary number of blood samples for antitoxin determination. When, in November 1944, our Institute was requested to undertake, free of charge, the collective immunization of the people living in a fairly well segregated dwelling quarter of Copenhagen (Ryparken), and also, at the request of the City Corporation of Copenhagen, the vaccination of the personnel working at the City Hall, the Institute agreed provided blood samples could be taken and a questionnaire submitted.

The following dosages were tested:

1. 2×1 ml. (corresponding to 25 Lf units per dose) with a four-week interval (the usual method employed in Denmark in the immunization of adults).
2. 2×0.5 ml. (i. e. 12.5 Lf units per dose) with a four-week interval.
3. 4×0.2 ml. (i. e. 5 Lf units per dose) with a one-week interval between the first and second injections, three weeks between second and third, and one week between third and fourth. This method had hitherto been selected when a milder course was advisable.
4. 3×0.2 ml. (i. e. 5 Lf units per dose) with a two-week interval between injections.
5. 2×0.2 ml. (i. e. 5 Lf units per dose) with a four-week interval between injections.

The last group was included in order to find out which interval was to be preferred, it having been suggested that an interval of one week between the two injections in Group 3 was so short that they might act as only one injection (Glenny & Südmersen, 1920—21). But as the immunization took place during a threatening epidemic, as already mentioned, it was considered indefensible to run the risk of immunizing Group 5 with only two small doses, and therefore this group was given an additional injection of 1 ml. two weeks after the second injection.

Blood samples for determining the natural antitoxin were taken from every person before inoculation. In the data which follow the antitoxin content of this blood sample will be called AT_0 . In the case of Groups 1, 3, 4 and 5, a blood sample (AT_1) was taken four weeks after the first injection; with Groups 1, 2, 4 and 5 another (AT_2) six weeks, and with Group 3 seven weeks, after the 1st injection, corresponding to two weeks after the last injection. A year later another blood sample (AT_3) was taken with all groups before the final injection. All the titrations were made according to Claus Jensen's intracutaneous rabbit method (1933). The dosage of the groups and the dates of the blood samples are shown in Fig. 1.

Groups 1, 3, 4 and 5 comprise the people living in »Ryparken«. The distribution of persons in the various groups was quite arbitrary. Group 2 (2×0.5 ml.) consists of some of the personnel of the Copenhagen City Hall.

Fig. 1.
Dosage and Date of Antitoxin Measurement.

Group	Weeks after first injection							
	0	1	2	3	4	5	6	c. 52
1	1.0 ml				1.0 ml			1.0 ml
2*)	0.5 "				0.5 "			0.5 "
3**)	0.2 "	0.2 ml			0.2 "	0.2 ml		0.2 "
4	0.2 "		0.2 ml		0.2 "			0.2 "
5	0.2 "				0.2 "		1.0 ml	0.2 "
Date of antitoxin measurement	AT ₀				AT ₁		AT ₂	AT ₃

*) No AT₁ measurement made in this group.

**) In this group AT₂ is measured in the 7th week, i. e. two weeks after the last of the two collective injections.

The Prophylactic.

The danish prophylactic is a highly purified toxoid adsorbed on to 10 per cent by volume of aluminium hydroxide (Schmidt & Hansen, 1933). The toxoid is purified by ultra-filtration through acetic acid-collodion membranes (Sierakowski & Zajdel, 1929; Scheibel, 1944). 1 in 10,000 merthiolate is added as an antiseptic to the prophylactic and after ampouling it is heated for half an hour at 56° to safeguard sterility.

The same batch of prophylactic was employed for all injections. It contained 25 Lf units per ml. The nitrogen content was 0.0016 mg. per Lf unit and the concentration of Al₂O₃ in the final prophylactic was about 2 mg. per ml. The antigenicity of the prophylactic was controlled on guinea-pigs by the method of Prigge (1935). A dilution corresponding to 50 Lf units per ml. was employed for the antigenicity test. At this dilution the prophylactic contained 130—320 protective units per ml. The injections were given subcutaneously in the regio supraspinata.

Natural Antitoxin.

First we give a survey of the distribution according to natural antitoxin within the various age groups.

The distribution of AT₀ proved to be the same in the two populations investigated. Therefore Table 2 comprises a total of 682 persons, 65 of whom had previously had clinical diphtheria. Of those who had not had diphtheria 61.2 % in the age group 15—29 years did not have measurable quantities of natural antitoxin in their serum. In the age group 30—49 years, the corresponding figure was 40.2 %. The probabi-

Table 2.
Distribution according to Natural Antitoxin.

Age	15—29				30—49				50—74					
AT ₀ units per ml	Previous Dipht.				Previous Dipht.				Previous Dipht.				Previous Dipht.	
	+		—		+		—		+		—		+	—
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	Total	Total
< 0.001	5	35.7	41	61.2	14	41.2	184	40.2	7	41.2	31	33.6		
0.001—0.01	3	21.4	5	7.5	6	17.6	58	12.7	2	11.8	16	17.4		
0.01—0.1	4	28.6	12	17.9	6	17.6	121	26.5	5	29.4	29	31.5		
0.1—1	2	14.3	7	10.4	7	20.6	83	18.1	3	17.7	14	15.2		
≥ 1	0	0.0	2	3.0	1	2.9	12	2.6	0	0.0	2	2.2		
Total	14		67		34		458		17		92		65	617

lity »P« of obtaining as great a deviation as that found can be calculated from the binomial law. When »P« is less than 5 % it is usual to reject the hypothesis under test. The calculation in question gives $P = 0.14$ % and hence the difference found is highly significant, and we may regard it as an established fact that there are more people with natural antitoxin in the age group 30—49 years than in the 15—29 year group. After the 30th year we can demonstrate no increase in the number of people with measurable antitoxin, the difference between 40.2 and 33.6 % not being significant ($P = 31.4$ %). People having more than 0.01 unit per ml. are usually considered to be protected against diphtheria. This limit leads to similar results: Under 30 years 68.7 % unprotected compared with 52.9 % in the age group 30—49; this gives $P = 1.8$ %, i. e. a significant difference, whereas the group 50—74 years has 51 % unprotected, or very nearly the same number as in the group 30—49 years (52.9 %). In an almost simultaneous investigation made in Copenhagen Ipsen (1946) found the same age limit for the increase in natural immunity, and he also found approximately the same distribution of protected and unprotected persons within the various age groups.

On comparing the group of those who have previously suffered from clinical diphtheria with the group of those who have not, we see that there is no difference between these two groups. The most marked deviation occurs in the age group 15—29 years among those containing <0.001 unit, viz. 35.7 % against 61.2 %. As the diphtheria group comprises only 5 persons, however, this difference is not significant, P being 14.2 %. If we put the limit at 0.01 unit the similarity of the two groups becomes even more conspicuous, the percentages in the three age groups being respectively 57.1 against 68.7, 58.8 against

52.9 and 53.0 against 51.0. Thus we can find no greater natural immunity among persons who previously (at least ten years earlier in the present investigation) had clinical diphtheria than among those who have not had the disease. This agrees with Strom's investigations in Oslo (1942) and Ipsen's in Copenhagen (1946), as well as with the observation often made earlier that clinical diphtheria by no means always causes production of antitoxin in convalescents (inter al. Karasawa & Schick, 1910; Otto, 1914; Rosling, 1929; Hamburger & Siegl, 1929; Madsen, 1939).

As was observed by Glenny & Südmersen in 1920 and by many others afterwards, there is a very pronounced difference between the effect of an injection of antigen in a sensitized and a non-sensitized individual. In a sensitized individual there will usually be more or less natural diphtheria antitoxin, and even infinitesimal quantities are of very great importance to the result of immunization. This is clearly illustrated by Table 3, which shows the antitoxin production in Group 1 after a single injection of 1 ml., partly in persons with no natural antitoxin and partly in persons with antitoxin in just measurable quantities.

Table 3.

AT ₀ units per ml	Number of individuals	AT ₁ units per ml
< 0.001	40	0.0087
0.001—0.01	9	28.2

As a consequence, our investigation of the dependence of the production of antitoxin on the dosage includes only persons with an AT₀ value of <0.001 unit per ml.

Sex, Age and Antitoxin Production.

In a number of diagrams on which the antitoxin titres were plotted against the age, and the sexes shown separately, we found no connection between the production of antitoxin and the sex or age. This applies as well to the AT₁ measurement as to the subsequent antitoxin determinations.

One of these diagrams is given here as an example.

This conforms to the investigations of Fulton et al. (1942) on children and young people up to 18 years of age, and to Seheibel & Bojlén's investigation (1947) on combined diphtheria-tetanus immunization of persons between 5 and 30 years.

In the sections which follow sex and age will therefore be disregarded.

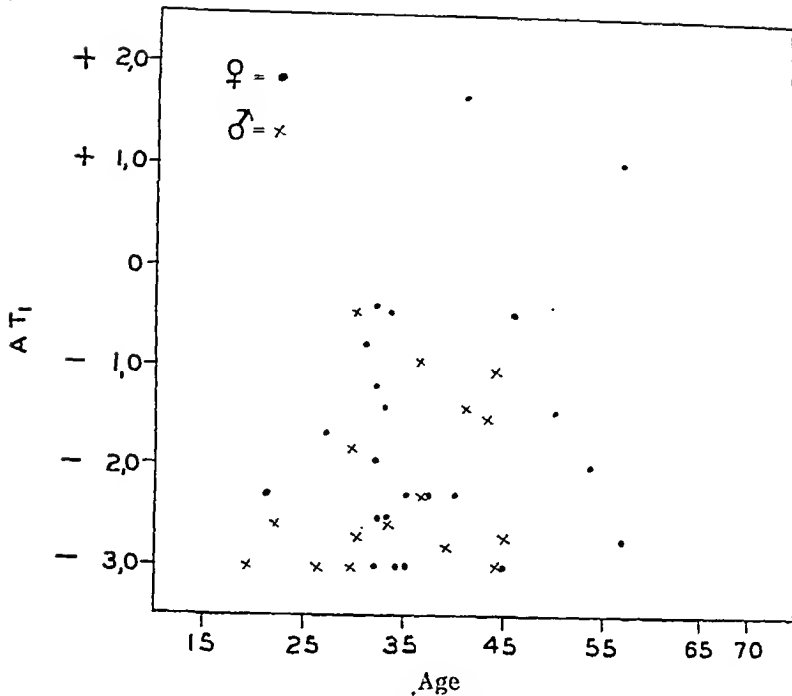


Fig. 2.

Showing the correlation between age, sex and antitoxin-production.

Dose, Interval and Antitoxin Production.

Table 4 summarizes the antitoxin titres (AT₁) four weeks after the first injection.

Table 4.
Titre of Antitoxin 4 Weeks after First Injection.
AT₁.

Group	Dose & Interval	No.	Average Titre units per ml		Protected (>0.01 units)	Standard Deviation
			Log. A. u.	Geom. mean	%	
1	1 ml	40	-2.06	0.0087	45.0	1.42
3	2×0.2 ml 1 week	43	-1.92	0.012	60.5	1.77
4	2×0.2 ml 2 weeks	27	-0.86	0.138	88.9	1.12
5	0.2 ml	40	-3.39	0.00041	32.5	2.35

Group 2 does not appear in this table because, as already stated, AT₁ was not measured on this group. Column 2 shows the quantity of prophylactic given prior to AT₁ and also the intervals between injections. In columns 4 and 5 the »mean titres« are shown. The logarithm

of the measurable titres seemed to follow a normal distribution.*) At this stage of the immunization, however, some of the titres were below the limit of what can be determined by the measuring technique, and accordingly the distribution became truncated. As a consequence, the simple mean titre must be corrected with regard to the truncation. The mean logarithmic titres of column 4 are corrected according to Hald (1945), and column 5 gives the corresponding anti-logarithms.

An examination of the titres at this early stage of the immunization reveals certain differences between the various dosages which do not appear in the final results. A statistical test showed that the means differed significantly. Presumably the deviation is due to the immunization methods. If however we first compare Groups 1 and 3, i. e. the ordinary routine immunization and the »small dose« method, we find, that they are very much alike as regards the mean titre. A statistical examination shows that the small differences found may be quite accidental; thus we get the same effect with two small doses given one week apart and aggregating less than half of the single larger dose, as with a single large dose in accordance with the experiments referred to by e. g. Park et al. (1932) and Povitsky (1932). If the interval is extended to two weeks, a comparison between Groups 3 and 4 shows a marked increase in the production of antitoxin. As this difference is 2.97 times the standard error it is clearly significant, the 5 %, 1 % and 0.1 % levels of the multiple being 1.96, 2.58 and 3.29 in sufficiently large samples.

As stated in the introduction, one of the objects of this work was to ascertain whether the two injections in Group 3 were given at so short an interval that they acted merely as one injection. Some light on this question may be thrown by a comparison of Groups 3 and 5. The geometrical titres for these two groups four weeks after the first dose are 0.012 and 0.00041 respectively; despite the very wide variation within each group, the difference proves to be significant ($t = 2.56$); thus there is a distinct effect from both injections in Group 3, though much less than when the interval is two weeks (Group 4). If finally a comparison is made between the two groups in which there was only one injection given (Groups 1 and 5), we find that the larger dose causes the greater production of antitoxin ($t = 2.41$). The fact that there is some dependence — though not particularly pronounced — between dose and antigenic effect was also found inter al. by Ramon & Nélis (1931), Kreitz (1932), Povitzky (1932), and Claus Jensen (1937), whereas certain other workers, including Paschlan (1939) and Schall & Lammert (1938), with the doses comprised in

*) The consequence is that all statistical tests are made on the basis of the averages and standard deviation of the logarithmic titres, which therefore are given in the tables along with the geometric mean employed in the practical appraisal.

their experiments found no relation between dose and antigenic effect. This effect depends largely upon whether the doses applied correspond to the steep part or to one of the flat parts of the dose-reaction curve; this may explain the diverging results.

Although the mean titre together with the standard deviation in principle gives a complete description of the state of immunity in a group, a direct statement of how many persons are considered protected, i. e. have produced more than 0.01 a. u. per ml., is rather illustrative. The relative number of protected persons is given in Table 4 column 6. A statistical test (the χ^2 test) shows that these percentages deviate significantly from one another. More detailed information is obtained by comparing the groups two by two. Groups 1 and 3 do not differ significantly ($P > 20\%$); this means that also in the degree of protection we can demonstrate no difference between two injections of 0.2 ml. administered at an interval of one week and a single injection of 1 ml. Neither do groups 1 and 5 differ significantly, which means that 1 ml. did not give a higher percentage of protection than 0.2 ml., although the larger dose, as the average titres show, causes a higher production of antitoxin. Evidently, the larger dose engenders a greater production of antitoxin only in those individuals where production starts at all after a single injection, but, what is more important in the practical prevention of diphtheria, does not bring more individuals to produce sufficient antitoxin.

The fact that two doses separated only by a week are superior in antigenic effect to a single dose of the same volume is also demonstrable with regard to the degree of protection, the difference between the percentages in Groups 3 and 5 being highly significant ($P = 0.75\%$). It is clear too, that an interval of two weeks is more favourable than one of one week, as Group 4 has 88.9 % protected compared with 60.5 % in Group 3 ($P = 1.4\%$).

Table 5.
Titre of Antitoxin 2 Weeks after the Last Injection.
AT₂.

Group	Dose & Interval	No.	Average Titre units per ml		Protected (> 0.01 units)	Standard Deviation
			Log. A. u.	Geom. mean	%	
1	2×1 ml 4 weeks	39	— 0.29	0.51	100.0	0.85
2	2×0.5 ml 4 weeks	76	— 0.41	0.39	97.4	0.93
3	4×0.2 ml 1-3-1 week	42	— 0.12	0.76	100.0	0.67
4	3×0.2 ml 2 weeks	30	— 0.48	0.33	93.3	0.97
5	2×0.2 ml 4 weeks	40	— 0.51	0.31	92.5	0.96

Table 5 shows the mean titres (AT_2) six weeks after the first injection. In column 2 we have entered the treatment preceding this antitoxin determination. As will be seen from Fig. 1, the blood sample for this test (AT_2) was taken two weeks after the last injection in all groups. At this stage of the immunization we found no difference in the effect of the different doses; the titres showed a normal distribution without truncation, and a statistical test revealed that the variation of the mean titres is no greater than what might have happened fortuitously. Even Group 5, which received only 2×0.2 ml. or a fifth of that in Group 1 is on a level with the other groups. The same is reflected in the protection percentages. In Groups 1 and 3, all those immunized have a titre of at least 0.01 unit per ml., and in the other three groups we find only respectively 2, 2 and 3 persons, corresponding to 2.6, 6.7 and 7.5 %, who have not exceeded this limit. These fluctuations are of such an order that obviously no importance can be attached to them; moreover, these seven persons all formed measurable antitoxin, the individual titres being 0.005 and 0.0063 unit in Group 2, 0.0075 and 0.008 unit in Group 4, and 0.002, .0.005 and 0.006 unit in Group 5.

It will thus be seen that the interdependence between dosage and antibody production which is demonstrable to some degree at the first stage of the immunization becomes effaced when more injections are given.

A comparison between AT_1 for Group 4 (Table 4) and AT_2 for Group 5 (Table 5) shows, in contrast to the conspicuous difference caused by increasing the interval from one to two weeks, no greater effect in the immunization by making the interval four weeks instead of two. The two mean titres are 0.14 and 0.31, a difference, which may be quite fortuitous ($t = 1.33$). Also, the protection percentages are very close together — 88.9 and 92.5; the 11.1 % unprotected in Group 4 represents three persons all containing some antitoxin: 0.003, 0.003 and 0.006 unit.

A striking point is the slight effect of the third dose in Group 4. Prior to this injection the mean titre was 0.14 and fourteen days later 0.33 unit per ml., an increase of only 2.4 times, whereas at the corresponding time in the other three groups where AT_1 was measured the antitoxin increase was 39.2, 29.3 and 520 times respectively. It seems as if the maximum effect was already obtained in Group 4 after the second dose, and that the third dose need not have been given, at any rate as far as the immediate result is concerned. This is supported by the standard deviation (Column 6 in Tables 4 and 5), Group 4 having a significantly smaller standard deviation at AT_1 than the other three groups, and practically the same deviation at AT_1 and AT_2 , whereas the other three groups record a much greater standard deviation at AT_1 than at AT_2 . This is in line with the general observation that the variability is greatest in the still incomplete phase of a process and declines towards the termination.

Does the Duration of Immunity depend upon Dosage?

As stated above, another blood sample for antitoxin determination (AT_3) was taken a year after the commencement of the immunization. The results are summarized in Table 6.

Table 6.
Titre of Antitoxin One Year after Immunization.
 AT_3 .

Group	Dose & Interval	No.	Average Titre units per ml		Protected (> 0.01 units)	Standard Deviation
			Log. A. u.	Geom. mean		
1	2×1 ml 4 weeks	25	— 1.19	0.065	100.0	0.61
2	2×0.5 ml 4 weeks	37	— 1.26	0.055	97.3	0.71
3	4×0.2 ml 1-3-1 week	26	— 1.15	0.071	96.2	0.75
4	3×0.2 ml 2 weeks	18	— 0.89	0.13	94.5	0.85
5	{ 2×0.2 ml 1×1 ml 4-2 weeks	20	— 1.13	0.074	100.0	0.46

Here again the titres have a normal distribution without truncation, and the mean titres and protection percentages are not significantly different. This apparently means that the duration of immunity is unaffected by the different methods of immunization employed within the period of the investigation. By means of guineapig tests Faragó in 1935 showed that the longer an antigenic action lasts, the less does the antitoxin titre fall. In this connection it is really surprising that the larger doses do not have a more stabilizing influence on the antitoxin level than the small doses. Group 1, for example, received more than three times as much antigen as Group 4. The explanation, however, must be that even with the smallest doses employed in this investigation we are on the flat part of the dose-response curve where even very marked increments in the antigen concentration have practically no effect.

Whether or not a dosage of twice 0.2 ml., corresponding to a total of 10 Lf units will suffice to ensure immunity to the same degree within the first year is a question which, unfortunately, is not answered by our experiments; at the time the investigation took place the epidemiological conditions were so serious that we considered it indefensible to rely on a dose which might perhaps prove to be too small, for which reason we gave this group (Group 5) a third injection of 1 ml. = 25 Lf units at the time when the AT_2 blood sample was taken. Another experimental series may possibly decide whether or not the result a year after immunization is independent of this extra dose.

Next, we have examined whether there is any relation between the AT_2 and AT_3 titres, i. e. between the titres six weeks and a year after the beginning of the immunization. For this purpose we divided the AT_2 titres into groups rising with the quotient 10, and calculated the mean titres for the various groups and the corresponding mean titres of AT_3 .

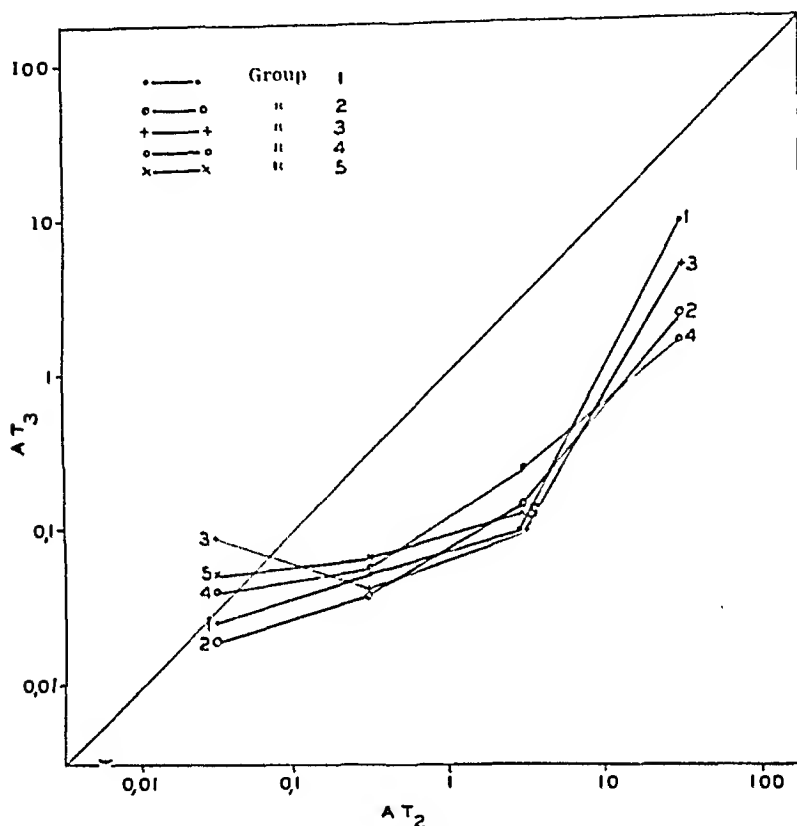


Fig. 3.
Showing the relation between AT_2 and AT_3 .

These two sets of mean titres are plotted against each other in Fig. 3, the AT_2 titres along the abscissa and the AT_3 titres along the ordinate. If the titres had not decreased the mean titres would have grouped themselves about the «identity line» drawn in the diagram. This is the case in the lowest group of titres (0.01—0.1), whereas there is a fairly uniform fall in the other three titre groups, which means that titres between 0.01 and 0.1 units remained unchanged during the period, whereas titres exceeding 0.1 unit deteriorated five to ten times. The conclusion to be drawn from this observation must be that it is more or less immaterial whether the titre immediately after immunization is 0.1—1 or ten times less as this difference after some time will dis-

appear. On the other hand, titres of more than 0.1—1 unit will also be correspondingly higher a year later.

Immediately after the AT_3 blood sample was taken, the immunization was completed with a booster dose for all who were available at this time. Group 1 received 1 ml., Group 2 0.5 ml., and Groups 3, 4 and 5 0.2 ml. However, as time went on it was difficult to persuade the whole population-group to continue; therefore, as antitoxic values were high before this injection (AT_3 titres, Table 6), the only blood samples taken two weeks later (AT_4) for judging the effect of the last injection were from persons whose AT_3 was lower than about 0.05 unit.

Table 7.
Titre of Antitoxin 2 Weeks after Boosting Dose.
 AT_4 .

Group	Dose	No.	Average Titre units per ml		Protected (> 0.01 units)	Standard Deviation
			Log. A. u.	Geom. mean		
1	1 ml	12	0.34	2.18	100.0	0.34
2	0.5 ml	18	0.16	1.45	100.0	0.40
3—4—5	0.2 ml	23	0.31	2.04	100.0	0.47

The means of these titrations are given in Table 7, from which emerges the fact that, likewise with the booster dose we are unable to demonstrate any correlation of antitoxin increase with size of dose.

As has been stated only persons whose AT_0 value was lower than 0.001 unit per ml. figure are recorded in the preceding investigation. However, the injections and the blood sampling were carried through with all who turned up for inoculation, regardless of their AT_0 value. The blood samples from the naturally immune were titrated later, though the titration was not carried beyond 90 units per ml. On examining the titres we found a marked difference between the antitoxin production of the naturally immune and of those who did not originally possess natural antitoxin, but who now, as a result of the first injections, had reached the same level of antitoxin as the naturally immune prior to vaccination. When these two groups were given the same antigen dose (corresponding to the first dose for the naturally immune and the second dose for the artificially immunized) the increase of antitoxin was many times greater in the naturally immune group (Fig. 4).

Along the abscissa are plotted the mean titres for AT_0 for the naturally immune and for AT_1 for the artificially immunized calculated as described for fig. 3, and along the ordinate the means of the corresponding AT_1 and AT_2 titres. The identity line indicates the placing of the points if there had been no increase in titre. As the curves showed the same course regardless of the dose, only one is drawn,

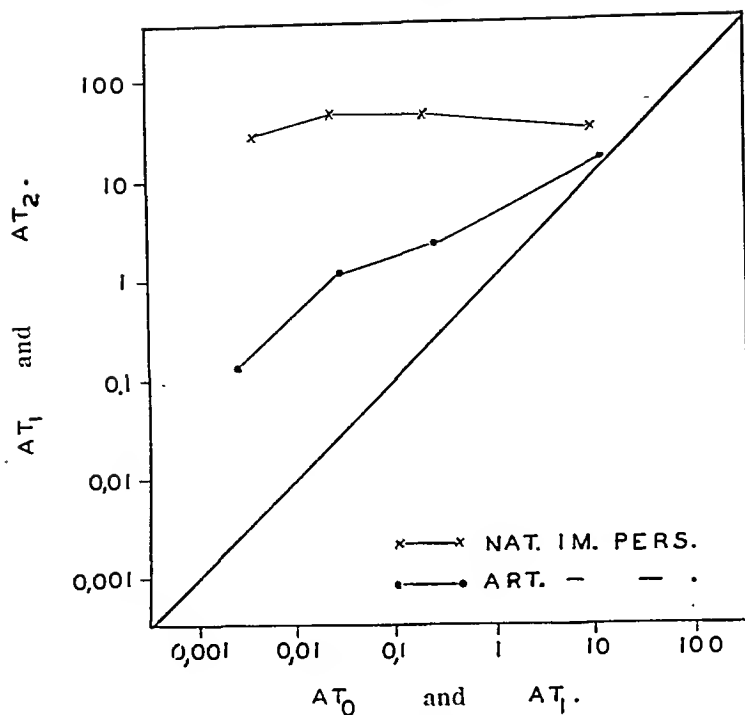


Fig. 4.

Showing the difference of antitoxin production in natural and artificial immunized individuals after an additional dose of prophylactic.

viz. that for Group 1. It will be seen from the diagram that there is a very marked difference in the effect of the injection, particularly as regards the lowest titres, although the level of antitoxin prior to immunization was the same for the two groups. For the higher titres the difference is less conspicuous, in conformity with what two of us have shown earlier (Bojlén and Scheibel, 1941). It may of course be maintained that the two series of mean titres are not directly comparable, as the AT_1 sample for the naturally immune was taken four weeks and the AT_2 sample for the artificially immunized two weeks after the injection. However the maximum antitoxin content after immunization of a sensitized organism is usually attained in one or two weeks (Glenny et al. 1920—21) so there is no reason to believe that the difference would have been less pronounced if the sample of the naturally immune group had also been taken two weeks after the injection.

As to the cause of this difference in antitoxin response we can at present only guess. On account of the wide distribution of diphtheria bacilli it would seem natural to imagine that under normal conditions all are exposed to the same risk of latent infection, but that only good antitoxin-producers react to this contact with a measurable antitoxin production. This differing ability to produce antitoxin might also

manifest itself under the much stronger antigenic action of an active immunization. It might also be possible that the time between sensitization and the next antigenic influence plays some role (Glenny et al. 1920—21).

Reactions.

In the present investigation it was impracticable to examine every person immunized and therefore we have had to rely on each individual's own observations.

It will always be difficult to frame a questionnaire that will cover briefly various types of symptoms and at the same time leave as little scope as possible for the individual opinions of patients and questioners, while simultaneously permitting of a classification of the information collected on anything like an objective basis.

We found that it served our purpose best to pay only little attention to vague symptoms, these often being determined more by the psyche of the patient than by the actual reaction; our questions were therefore devised to collect information on more well-defined local symptoms such as tenderness, infiltration and muscular pain, and on general symptoms like increased temperature.

After collecting and examining the answers we have set up three degrees of reaction, designated 0, 1 and 2.

0 represents persons who either had no reaction or only insignificant local and general reactions, e. g. very slight tenderness and infiltration as well as very brief and very slight temperature increase ($<38.5^{\circ}$ less than one day) when the latter was unaccompanied by other symptoms.

1 covers persons with slight local and general discomfort such as moderate infiltration at the site of injection, moderate tenderness and brief but marked temperature increase ($>38.5^{\circ}$ not more than 1 day).

2 designates pronounced local and/or general symptoms with confinement to bed or incapacity for work.

It may be mentioned in passing that there were one case of disablement for 10 days, one for 7 days, three for 6 days and the remainder for 2 to 4 days. No known pathological picture was found in any instance, nothing but only transient complications.

This grouping was found to be practical, as it differentiates between reactions which were a real inconvenience (Reaction 2) and those of more secondary importance.

Experiments in having different people working independently to classify the material along these lines showed very small deviations, so that we seemed to have secured an objective appraisal of the information collected.

In the following sections we shall confine ourselves to the reactions after the first injection. The estimation of frequency of reactions after the subsequent injections was made very difficult by the

fact that many of those who had strong reactions after the first injection simply did not turn up for the next. For this reason the fall in reaction frequency and degree observable from a direct count must be viewed with all reserve. We should add, however, that no increase in reaction percentage from the first to the second injection was found even if it be assumed that all persons, who had a severe reaction after the first injection and who stayed away from the following would have reacted strongly on this too. Among those receiving two injections there proved to be a 35—50 % chance of having disagreeable reactions after the second injection if the reaction had been strong after the first, and only about a 10 % chance of a reaction if there had been none after the first injection.

As we are concerned here solely with the reactions after the first injection, we decided that Groups 3, 4 and 5 which received the same dose, 0.2 ml. at the first injection should be investigated together as one group: 3 A; this gives us only three groups to analyse viz. Group 1 receiving 1 ml., Group 2 receiving 0.5 ml. and Group 3 A receiving 0.2 ml.

Table 8.
Distribution according to Reactions.

Group	Degree of reaction						Total No.
	0		1		2		
	No.	%	No.	%	No.	%	
1	99	76.7	9	7.0	21	16.3	129
2	152	82.2	7	3.8	26	14.1	185
3 A	311	84.5	21	5.7	36	9.8	368
							682

In Table 8 these groups are segregated according to degree of reaction. It will be seen that Reaction 1 occurs with varying frequency and apparently independently of the doses, whereas Reaction 2 seems to decrease somewhat when the dose is reduced to 0.2 ml. The difference in the frequency of Reaction 2 with the two large doses and with the smallest dose is only just on the border of significance ($P = 5\%$). A simple appraisal of the dependence of the reactions on the dose cannot, however, be made from such an undiversified grouping, where other factors possibly having a bearing upon the reactions may have screened or distorted the result. It was therefore necessary to attempt the clarification of the significance of such of the various factors as could be investigated within the scope of the present material, and to throw some light upon their interrelation.

Reactions and Natural Antitoxin.

There can scarcely be any doubt that those reactions which, despite the use of highly purified prophylactics freed of every trace of specific diphtheria toxin, nevertheless occur in some of the inoculated persons, must to a great extent be allergic in nature, and that the cause of the sensitization must be sought chiefly in an usually unobserved contact with diphtheria bacilli. This was demonstrated e. g. by Park (1922), Zoeller (1925) and Zingher (1923). As was shown by Otto in 1914 and Dudley, May & O'Flynn in 1934, this contact also has another result, namely the production of natural antitoxin. The obvious course to adopt is therefore first to look for some connection between the natural antitoxin and the reactions. It has previously been observed that there is such a connection. It is true that Schall & Lammert (1938) could demonstrate no regular dependence between AT_0 and the reactions, although most reactions occurred among persons with AT_0 of over 0.03 unit. In an investigation covering infant immunization Paschlaue (1939) saw reaction in all children who recorded much natural antitoxin (> 0.03 unit per ml.). On the other hand, reactions also occurred in children who had no natural antitoxin. After the immunization of probationer-nurses Plum (1939) observed most reactions in those with natural antitoxin. Fulton, Taylor, Moore, Wells & Wilson (1942) found significantly more reactions among Schick-negative than among Schick-positive children.

As already mentioned, it is usual to look upon people with more than 0.01 unit per ml. as being immune against diphtheria. After having ascertained that no difference could be found in the occurrence of reactions in persons with no measurable antitoxin, i. e. less than 0.001 unit, and in those with between 0.001 and 0.01 unit, we considered it reasonable to use the latter titre, which is of a certain practical importance, as the dividing limit.

In Table 9 the material is divided according to natural antitoxin and degree of reaction.

Table 9.
Influence of Natural Antitoxin on Reaction.

Group & Dose	AT ₀ < 0.01							AT ₀ > 0.01						
	Degree of reaction						Total	Degree of reaction						Total
	0		1		2			0		1		2		
	No.	%	No.	%	No.	%		No.	%	No.	%	No.	%	
1 1 ml	56	90.3	1	1.6	5	8.1	62	43	64.2	8	11.9	16	23.9	67
2 0.5 ml	97	89.8	3	2.8	8	7.4	108	55	71.4	4	5.2	18	23.4	77
3 A 0.2 ml	182	90.0	9	4.5	11	5.4	202	129	77.7	12	7.2	25	15.1	166

On comparing the right and left halves of the table it becomes obvious that, as in the earlier investigations just quoted Reaction 2 has its highest frequency among persons with $AT_0 > 0.01$ at all three doses.

The differences are significant, the P-calculation giving the following values:

	$AT_0 < 0.01$	$AT_0 > 0.01$	P:
Group 1	5/62 = 8.1 %	16/67 = 23.9 %	3.0 %
» 2	8/108 = 7.4 %	18/77 = 23.4 %	0.5 %
» 3 A	11/202 = 5.4 %	25/166 = 15.1 %	0.4 %

The weaker reactions seem to follow the same line but much less markedly. In Group 1 alone the difference is significant ($P = 2.8\%$), whereas the other two groups record quite insignificant differences. Whether this accumulation is really due to the natural antitoxin, or whether the picture will be changed if the material is divided according to other factors which the scope of this work will permit us to investigate, are questions which cannot be answered until the bearing of these factors on the reactions is elucidated. In the following, the connection between the AT_0 value and the occurrence of the reactions will be employed as a working hypothesis. The final adjudication will be reverted to later.

Reactions and Age.

It has previously been mentioned as a common observation that older children and adults have more severe and more frequent reactions after diphtheria immunization than infants — see e. g. Paschla (1939), Lereboullet & Gournay (1929) and Ramon (1944). By means of Moloney's test, Moloney, Fraser & Fraser (1929) find that hypersensitivity increases with age. Underwood (1935) does not consider that age as such means anything to this hypersensitivity, but thinks it due to the fact, that the number of naturally immune increases with age.

We have examined the question of whether, between the age groups comprised in this work, there is any difference in the reaction frequency when the AT_0 is considered simultaneously. For this purpose we have divided the material into three age groups: 15—29, 30—49, and 50—74 years. In Table 10 these age groups are set up according to degree of reaction.

If we take the left half of the table ($AT_0 < 0.01$) it is obvious that there is no evident increase in reactions with rising age; this applies to both Reaction 1 and Reaction 2. In the first place, the differences in reaction percentages are only small in comparison with the number of observations and in the second place, most reactions occur sometimes in one age group and sometimes in the other. The same is observed in the two youngest age groups among persons with $AT_0 > 0.01$

Table 10.
Influence of Age on Reaction.

Group & Dose	Age	AT ₀ < 0.01							AT ₀ > 0.01						
		Degree of reaction						Total No.	Degree of reaction						Total No.
		0		1		2			0		1		2		
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%	
1. 1 ml	15 - 29	9	100,0	0	0,0	0	0,0	9	4	66,7	0	0,0	2	33,3	6
	30—49	42	89,4	1	2,1	4	8,5	47	29	59,2	7	14,3	13	26,5	49
	50—74	5	83,3	0	0,0	1	16,7	6	10	83,3	1	8,3	1	8,3	12
	Total	56	90,3	1	1,6	5	8,1	62	43	64,2	8	11,9	16	23,9	67
	2. 0,5 ml	15—29	14	93,3	1	6,7	0	0,0	15	5	83,3	0	0,0	1	16,7
30—49		61	88,4	2	2,9	6	8,7	69	32	60,4	4	7,5	17	32,1	53
50—74		22	91,7	0	0,0	2	8,3	24	18	100,0	0	0,0	0	0,0	18
Total		97	89,8	3	2,8	8	7,4	108	55	71,4	4	5,2	18	23,4	77
3. A 0,2 ml		15—29	27	90,0	1	3,3	2	6,7	30	13	86,7	0	0,0	2	13,3
	30—49	130	89,0	8	5,5	8	5,5	146	96	75,0	10	7,8	22	17,2	128
	50—74	25	96,1	0	0,0	1	3,9	26	20	86,9	2	8,7	1	4,4	23
	Total	182	90,0	9	4,5	11	5,4	202	129	77,7	12	7,2	25	15,1	166

(the right half of the table). Reaction 2 occurs with fluctuating frequency and Reaction 1 apparently is most frequent in the age group 30-49; but the difference, having regard to the few observations in the youngest group, is of such a size that obviously it cannot be credited with any significance.

The situation looks different when we consider the frequency of Reaction 2 in the two oldest age groups with $AT_0 > 0.01$. Here we see a striking decrease of Reaction 2 in the oldest group. In Group 1 we find $13/49 = 26.5\%$ in the age group 30-49 compared with $1/12 = 8.3\%$ in the age group 50-74 years; in Group 2 $17/53 = 32.1\%$ against $0/18 = 0.0\%$, and in Group 3 A $22/128 = 17.2\%$ against $1/23 = 4.4\%$. This gives the following P values: 26 %, 0.7 % and 12.5 % respectively, which means that we find a significant difference only in one group. This significance has been examined with the aid of the binomial law, resulting in a probability of 2 % of getting $P < 0.7\%$ in the course of three comparisons; in other words, if age exerted no influence on the reaction, a P as low as that arrived at would be found

in only 2 out of 100 cases. This slight probability, combined with the fact that the difference in all three groups has the same trend, i. e. towards a lower reaction percentage in the oldest age group, makes it justifiable to consider the fact demonstrated that the intensity of reaction decreases when a certain age has been reached, even when an individual according to his AT_0 belongs to the group in which most of the severe reactions may be anticipated.

With regard to Reaction 1, we can find no difference between the two age groups. In Group 1 the figure is 14.3 % in the age group 30—49 years against 8.3 % in 50—74 years. For Group 2 the corresponding figures are 7.5 % against 0 %, and for Group 3 A 7.8 % against 8.7 %. The difference between the first two groups might appear conspicuous; but having regard to the small denominators it is immediately seen that the differences are of no significance.

As we can find no increase of the weak reactions in the oldest age group simultaneously with the observed decrease in the strong reactions, it is therefore not only the reaction intensity that falls in the oldest age group, but the reaction frequency as well.

Reactions and Dose.

As was stated in the introduction, we naturally endeavoured to avoid the disagreeable reactions among adults by reducing the dose. To our knowledge, however, there are only very few investigations aimed more systematically at making comparisons between different doses and their influence upon the reactions. In the result of mass immunization against diphtheria, published in Canada and England, the hypersensitives were primarily sorted out by means of the Moloney or the Schick test and then given small doses, or none at all (Moloney & Fraser, 1927; Moloney et al. 1928; Burke, 1930; Fraser, 1931; McKinnon, Ross & Defries, 1931; Underwood, 1935). These investigations contain only little control material to show how a usual dose would have acted — which of course is only natural, as it would scarcely be proper to inoculate people in whom there was reason to anticipate reactions with the usual dose. But when, as in the present investigation, no hypersensitivity test is made prior to immunization, there is a chance of finding out whether a reasonable reduction of the dosage is reflected in the reaction frequency. Ramon, Timbál & Nélis (1933) claim to have seen a slight increase in reactions when the titre of the anatoxin rose from 16 to 20 Lf units per ml. Claus Jensen (1937) observed rising reaction figures with increasing doses, and Schall et al. (1938) are inclined to think that there may be some interdependence between size of dose and number of reactions. Paschla (1939) found that a dose variation from 0.2 to 1 ml has no influence on reactions among infants, but some among school children, where indeed he found an increase of reactions when the dose was

raised from 0.2 to 0.3 ml. In none of these investigations, however, was attention paid to other factors relevant to the occurrence of reactions, for example, the AT_0 and the age.

Having shown that the age groups 15—29 and 30—49 do not differ as to reaction when they have the same AT_0 , we have combined them into one age group, 15—49, in Table 11.

Table 11.
Influence of Dose on Reaction.

Age	Group & Dose	AT ₀ < 0,01								AT ₀ > 0,01							
		Degree of reaction						Total No.	Degree of reaction						Total No.		
		0		1		2			0		1		2				
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%			
15—49	1 1 ml	51	91,1	1	1,8	4	7,1	56	33	60,0	7	12,7	15	27,3	55		
	2 0,5 ml	75	89,3	3	3,6	6	7,1	84	37	62,7	4	6,8	18	30,5	59		
	3 A 0,2 ml	157	89,2	9	5,1	10	5,7	176	109	76,2	10	6,9	24	16,8	143		
50—74	1 1 ml	5	83,3	0	0,0	1	16,7	6	10	83,3	1	8,3	1	8,3	12		
	2 0,5 ml	22	91,7	0	0,0	2	8,3	24	18	100,0	0	0,0	0	0,0	18		
	3 A 0,2 ml	25	96,1	0	0,0	1	3,9	26	20	89,9	2	8,7	1	4,4	23		

A comparison between Groups 1 and 2 shows that the frequency and degree of the reactions are much the same for these two doses. The oldest age group does exhibit an apparently higher reaction percentage after 1 ml than after 0.5 ml; but again it is obvious that the differences are immaterial. Accordingly, as a decrease in the dose from 1.0 to 0.5 ml is not reflected in the reaction, we have pooled both groups into Groups 1 + 2 of Table 12 and undertaken the comparison with this simplified table as a basis.

It stands out clearly from Table 12 that the lowest dose causes an appreciable reduction in the reactions in only one group; on the other hand, this particular group lies within the population group that is threatened most by strong reactions, viz. persons with an AT_0 of over

Table 12.
Influence of Dose on Reaction.

Age	Group & Dose	AT ₀ < 0,01							AT ₀ > 0,01						
		Degree of reaction						Total No.	Degree of reaction						Total No.
		0		1		2			0		1		2		
		No.	%	No.	%	No.	%		No.	%	No.	%	No.	%	
15—49	1 + 2 1 ml & 0,5 ml	126	90,0	4	2,9	10	7,2	140	70	61,4	11	9,6	33	29,0	114
	3 A 0,2 ml	157	89,2	9	5,1	10	5,7	176	109	76,2	10	6,9	24	16,8	143
50—74	1 + 2 1 ml & 0,5 ml	27	90,0	0	0,0	3	10,0	30	28	93,3	1	3,3	1	3,3	30
	3 A 0,2 ml	25	96,1	0	0,0	1	3,9	26	20	89,9	2	8,7	1	4,4	23

0.01 unit in the age group 15—49 years. Here the large doses cause Reaction 2 in 29 % and the small dose in 16.8 %. The probability of such a difference is only 1.5 %; consequently by diminishing the dose we actually reduce the strong reactions.

Reactions and Sex.

In order to ascertain whether reactions are more frequent in one sex than in the other, we have separated the groups of Table 12 according to sex and set them up in Table 13.

For Reaction 2 there is only one instance of real difference, in Group 1 + 2, $AT_0 < 0.01$, 15—49 years, where there are 2.5 % males and 13.1 % females. In itself this difference is significant, for P is 2.1 %. But as we find only one significant difference in eight comparisons, and as the other seven show only slight differences, and even then with varying signs, we cannot credit this one significant difference with any importance, but must conclude that Reaction 2 is equally frequent in both sexes. The same applies to Reaction 1. Here again we see once only a more marked difference, i. e. in Group 3 A, $AT_0 > 0.01$, 15—49 years, where there are 3 % reacting males against 10.5 % reacting females, but in this case the difference is not significant ($P = 10.5$ %).

Table 13.
Influence of Sex on Reaction.

Group & Dose	Age	Sex	AT ₀ < 0,01								AT ₀ > 0,01							
			Degree of reaction						Total No.	Degree of reaction						Total No.		
			0		1		2			0		1		2				
			No.	%	No.	%	No.	%		No.	%	No.	%	No.	%			
1 + 2 1 ml & 0,5 ml	15—49	♂	75	94,9	2	2,5	2	2,5	79	30	56,6	6	11,3	17	32,1	53		
		♀	51	83,6	2	3,3	8	13,1	61	40	65,6	5	8,2	16	26,2	61		
	50—74	♂	12	92,3	0	0,0	1	7,7	13	18	94,7	0	0,0	1	5,3	19		
		♀	15	88,2	0	0,0	2	11,8	17	10	90,9	1	9,1	0	0,0	11		
3 A [0,2 ml]	15—49	♂	63	86,3	5	6,8	5	6,8	73	56	83,6	2	3,0	9	13,4	67		
		♀	94	91,3	4	3,9	5	4,9	103	53	69,7	8	10,5	15	19,7	76		
	50—74	♂	14	100,0	0	0,0	0	0,0	14	12	80,0	2	13,3	1	6,7	15		
		♀	11	91,7	0	0,0	1	8,3	12	8	100,0	0	0,0	0	0,0	8		

Taking it on the whole, no difference can be found in this investigation between males and females with regard to the frequency and degree of the reactions.

Reactions and Former Diphtheria.

As stated on page 325, we cannot find any difference in the AT₀ titre of persons who have previously had clinical diphtheria and those who have not. As hypersensitivity to the diphtheria antigen does not — despite some accordance — run parallel to AT₀, it was natural to examine whether an earlier diphtheria, though perhaps not leaving traces in the antitoxin production, might not have left a state of allergy, the manifestation of which would be that persons who had once had clinical diphtheria would have reactions more frequently than others after diphtheria immunization. In investigations not taking into account AT₀ or other factors, Claus Jensen (1937) and Wohlfeil (1938) observed most reactions among children who already had had diphtheria. In Table 14 we have divided the data of Table 12 into + and — earlier diphtheria, in this case, however, restricting ourselves to Reaction 2.

When AT_0 is less than 0.01, we find everywhere the highest reaction frequency among persons who have had diphtheria. As there regarding the other factors here examined is no demonstrable difference in the occurrence of reactions when the AT_0 is lower than 0.01, it is permissible to combine the various groups. This gives $8/37 = 21.6\%$ cases of reaction 2 among persons who have had diphtheria, and $16/335 = 4.8\%$ among those who have not. This difference being highly significant ($P = 0.02\%$), we may draw the conclusion that an attack of clinical diphtheria, even when dating several years back (in this in-

Table 13.
Influence of Previous Diphtheria on Reaction 2.

		$AT_0 < 0.01$		$AT_0 \geq 0.01$	
Group	Age	+ Diphtheria	- Diphtheria	+ Diphtheria	- Diphtheria
1 + 2	< 50 years	2/8 = 25.0%	8/132 = 6.1%	3/13 = 23.1%	30/101 = 29.7%
	> 50 "	1/6 = 16.6%	2/24 = 8.3%	1/6 = 16.6%	0/24 = 0.0%
3 A	< 50 "	4/20 = 20.0%	6/156 = 3.8%	1/7 = 14.3%	23/136 = 16.9%
	> 50 "	1/3 = 33.3%	0/23 = 0.0%	0/2 = 0.0%	1/21 = 4.8%

vestigation at least ten years) will often leave a hypersensitivity, manifested in rather strong reactions when diphtheria antigen is injected. Thus, clinical diphtheria is a relevant factor in the occurrence of reactions, and it therefore became necessary to see whether omission of persons who had had diphtheria made any change in the relation found of age and dose to the reactions. This proved not to be the case.

For persons with an AT_0 of 0.01 or more the reaction frequency seems to be very nearly the same for the two categories. Only once do we find the highest reaction percentage among persons who had diphtheria, and this difference cannot lay claim to any significance owing to the small figures. In this part of the investigation, where both age and dose influence the reactions, one is precluded from calculating a pooled reaction frequency. The few observations make superfluous a calculation of P within the various groups of comparison, it being obvious that there are no significant differences. A simple inspection of the values makes it tempting to believe that an earlier diphtheria does not increase the chances of obtaining reactions when one has an AT_0 higher than 0.01 a. u.; in other words, that a clinical diphtheria might perhaps not leave more hypersensitivity than a latent infection which is reflected in an antitoxin production of a certain degree.

Reactions and Antitoxin Production.

Here and there in the literature one encounters the assumption that reactions are synonymous with good antitoxin production (Bousfield & King-Brown, 1938; Schall, 1938; Schall et al. 1938; Paschla, 1939). Ramon (1926) claims to have made this observation when immunizing horses, which led to addition to the antigen of nonspecific local reaction-inducing substances such as tapioca, which is often used in the preparation of antitoxic sera. Among children Schall et al. (1938) found good antitoxin production in all cases where local reaction appeared, including cases in which the AT_0 was lower than 0.03 a. u. However, they failed to take into consideration the size of the dose (in their case varying from 0.2 to 2 ml) or earlier attacks of diphtheria which, as just stated, may be of importance for the appearance of reactions. Plum (1939) on the other hand found no relationship between local reactions and antitoxin production. In the present material only Group 1 and a part of Group 3 A (Group 5) are suitable for a discussion of this question, as the other groups either have no AT_1 determination or have had several injections between AT_0 and AT_1 . As antitoxin production is highly dependent on AT_0 , even at AT_0 values as low as between 0.001 and 0.01 unit, we have here had to make use of 0.001 unit as the limit of division, persons with an earlier diphtheria, however, being omitted as this factor has shown to be relevant (p. 343).

Table 15.
Influence of Antitoxin Production on Reactions.

$AT_0 < 0.001$				
AT_1	Degree of reaction			Total
	0	1	2	
< 0.01	54	1	0	55
> 0.01	31	0	3	34

Most reactions and all the severe ones occur in the group showing the greatest antitoxin increases. The frequency of Reaction 2 in the two groups is 0 % compared with 8.8 %; this gives $P = 5.3$ %, or just on the border of significance. Thus, the present data do not give a clear answer to the question mentioned about a relation between the reactions and the antitoxin production. On attempting an examination of the same question among persons with $AT_0 > 0.01$, matters become more complicated, for we must consider the different dosage in the two groups and moreover place the various AT_1 values in relation to their respective AT_0 values in order to arrive at a picture

of the real titre increase. The consequence is that the figures in the various groups become so small that they evade judgment of any kind. For this group therefore we leave the question open.

Concluding Remarks on the Importance of Natural Antitoxin to Reactions.

Having now established the fact that dose, age and earlier clinical diphtheria all have an influence on reactions, whereas sex seems to have no bearing on them, we shall once more turn to the interdependence between reaction and AT_0 with due consideration to the above factors. For this purpose the material is divided according to dose, age and clinical diphtheria with the reduced groups which the investigation has shown to be justifiable. The analysis comprises Reaction 2 alone.

Table 16.
Influence of Natural Antitoxin on Reaction 2.

Group		+ previous Diphtheria		- previous Diphtheria	
		$AT_0 < 0.01$	$AT_0 > 0.01$	$AT_0 < 0.01$	$AT_0 > 0.01$
1 + 2	< 50 years	2/8 = 25.0 %	3/13 = 23.1 %	8/132 = 6.1 %	30/101 = 29.7 %
	> 50 "	1/6 = 16.6 %	1/6 = 16.6 %	2/24 = 8.3 %	0/24 = 0.0 %
3 A	< 50 "	4/20 = 20.0 %	1/7 = 14.3 %	6/156 = 3.8 %	23/136 = 16.9 %
	> 50 "	1/3 = 33.3 %	0/2 = 0.0 %	0/23 = 0.0 %	1/21 = 4.8 %

The figures in this table are the same as those in Table 14, though to facilitate the final comparisons we tabulate them in a different manner.

If there is a history of diphtheria, the reactions seem to occur independently of AT_0 , but the observations are so few, that we cannot attach much weight to this conclusion.

Among persons who have not had diphtheria there is no important difference in the reaction frequency in the oldest age group; the few reactions in this group seem to be independent of AT_0 . In the age group < 50 years, however, we recognize the same picture as in Table 9: most of the severe reactions occurred among persons with $AT_0 > 0.01$. In this selected material the differences are highly significant, P being only 0.001 % for Group 1 + 2 and 0.03 % for Group 3 A (compare page 337).

Whether the role played by the natural antitoxin is due merely to its running parallel with a bacterial hypersensitivity, or whether the interaction between antitoxin and injected antigen may cause or perhaps intensify any complications, are questions that so far have not been answered. If it is true that AT_0 has nothing to do with the occur-

rence of reactions once there has been clinical diphtheria, this might indicate that the bacterial hypersensitivity is the deciding factor. In this connection, however, Neill, Sugg & Richardson (1931) showed, that guinea-pigs inoculated with homologous antitoxic serum freed from bacterial antibodies suffer from anaphylactic shock when subsequently injected with purified diphtheria toxin intravenously.

If reactions can be caused by the antibody-antigen combination here involved one would expect an increase of reactions with increasing AT_0 . An examination of this possibility however gave a negative result, no relationship being found between Reaction 2 and AT_0 values, when the latter were divided into groups rising with the quotient of 10. It should be stressed, that this examination must be taken with some reserve, owing to the groups being small when the reactions are distributed over various AT_0 values.

Summary.

The relationship of antitoxin production to dose of prophylactic and interval between injections has been investigated. If the results are judged according to a single injection, the antitoxin production depends to some extent on the size of the dose, 1 ml. causing a greater production of antitoxin than 0.2ml., when judged from the mean values, but not a higher percentage of protected subjects, i. e. individuals having more than 0.01 unit per ml. in their blood.

Two doses of 0.2 ml. administered a week apart gave the same degree of immunity as regards both antitoxin production and percentage of protected subjects as a single dose of 1 ml., and a better result than a single dose of 0.2 ml.

Two doses of 0.2 ml. with a fortnight's interval resulted in a much greater antitoxin production and percentage of protected subjects than the same doses given a week apart, whereas there was no demonstrable difference in response when the interval was increased from two to four weeks.

If the results are appraised at a later stage, when more injections have been given, there appeared to be no dependence of the antibody production on the antigen doses employed.

The antitoxin titres 1 year after immunization were independent of the procedures used.

The increase of antitoxin after a boosting dose a year later was independent of the doses used.

With regard to reactions, the investigation has shown that they were influenced by the following factors: Natural antitoxin, history of diphtheria, dose of prophylactic and age.

Among people having less than 0.01 units pr. ml. prior to the immunization we found about 5 % severe reactions occurring regardless both of whether the dose was 1 or only 0.2 ml. and of age but chiefly amongst those who have had diphtheria.

Among people containing 0.01 unit or more per ml. prior to the immunization and being under fifty years of age we found about 30 % severe reactions after 1 or 0.5 ml and about 17 % after 0.2 ml. These reactions occurred presumably regardless of whether there was a history of diphtheria or not. Among people containing 0.01 unit or more per ml., and being over fifty years of age we found about 5 % severe reaction regardless of the doses here used.

If one has no previous knowledge at all regarding the occurrence of the various relevant factors in a population, one must thus anticipate a minimum of 5 % and a maximum of 30 % severe reactions when 1 or 0.5 ml. is given and of 5 % and 17 % respectively when 0.2 ml. is given. With a representations of the factors as in the present material the reaction percentage after the first injection averages 15.0 % when the dose is 1 or 0.5 ml. and 9.8 % when it is 0.2 ml.

The reaction frequency for the two sexes is the same. Correlation can be observed between reaction and antitoxin production, but the material is too small to permit of final conclusions on this point.

CONCLUSIONS

In the introduction, we stated four questions as the principal object of this work. We shall repeat these questions here: 1) Does immunization with four doses of 0.2 ml. give the same result as immunization with two doses of 1 ml. although the total antigen content is thereby reduced from 50 to 20 Lf units? 2) In cases where it is necessary to employ a milder course will it suffice by changing the intervals to give three injections of 0.2 ml. instead of four? 3) Can the usual routine dose be reduced from 1 ml. to 0.5 ml?; and finally 4) Do the reactions decrease according to the size of the dose?

The first three questions can be answered straight away in the affirmative, no difference in immunization result having been demonstrable after the various procedure. The differences revealed by the AT_1 titration are only of theoretical interest, provided that the vaccination is carried out *lege artis*. As to question 4, we found that severe reactions do diminish with decreasing doses, but that it is only demonstrable in the group most likely to have reactions, and only, when the dose is reduced to 0.2 ml. Accordingly our ordinary routine method will hereafter be 2×0.5 ml., i. e. 2×12.5 Lf units with a four-week interval, and a year later another 0.5 ml. When smaller doses are indicated, 3×0.2 ml., i. e. 3×5 Lf units at two-week interval will be given, and a year later another 0.2 ml.

It would be quite proper, in mass immunization too, to give merely 0.2 ml. for the boosting dose; however, for practical reasons for the present we prefer to retain the same dose for all three injections, and only in cases where the reactions after the foregoing injections indicate it, 0.2 ml. should be given.

The investigation gives furthermore some reason to believe, that the dose for mass immunization could be diminished even to 2×0.2 ml. (2×5 Lf units) followed a year later by a third injection of 0.2 ml. This would indeed be very desirable as the decrease in reactions first will be noticeable at this dose. However as the group starting with this dosage for reasons already mentioned was given an additional injection of 1 ml. further investigation have to be undertaken before this question can be settled.

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ON THE PREPARATION OF A FORMALINIZED ALUMINIUMHYDROXIDE VACCINE WITH THE MURINE S. K. POLIOMYELITIS STRAIN (JUNGEBLUT & SANDERS)*)

By *A. Frantzen.*

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In the following an experiment in preparing a formalinized aluminiumhydroxide vaccine with the S.K. strain of Jungeblut & Sanders from fluid tissue culture is reported.

This strain possesses a high and constant virulence to mice and therefore is very suitable for experiments of this kind which require exact titration. On the other hand, it is doubtful whether serologically it is so closely related to human types of virus that it will be suitable for the prophylactic vaccination of man.

This being so, our experiments must be regarded as models; the experience gained from them will only be put to practical use when it is possible in vitro to cultivate a virus exhibiting indubitable serological conformity with the human types of virus against which immunization is desired.

One of the claims we must make of a vaccine to be used for the prophylactic vaccination of man is low toxicity. A vaccine prepared directly from the brain or spinal cord of infected animals will satisfy this requirement only after very thorough processes of purification. But we have a more suitable primary material when the virus can be cultivated in a fluid tissue culture and the virus is segregated from the supernatant fluid. In such a fluid one can find virus in relatively high concentrations, although there is no measurable protein.

In 1940 Jungeblut & Sanders (2, 3) cultivated the murine S.K. poliomyelitis virus in several passages. The medium was embryonic

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mouse brain in a fluid consisting of serum ultrafiltrate and a modified Tyrode fluid described by Simms & Sanders (5). The composition of this fluid is given in table 1.

Table 1.
*Composition of the salt solution used
for tissue culture.*

	mM per Litre
NaCl	137.0
KCl	2.7
CaCl ₂	1.0
MgCl ₂	1.0
NaHCO ₃	12.0
Na ₂ HPO ₄	1.5
Dextrose	5.5

Jungeblut & Sanders subsequently demonstrated that cultivation is also possible without serum ultrafiltrate. This is the method employed in the present experiments.

Cultivation proceeds in 50 cc. Erlenmeyer flasks containing 10 cc. fluid. Owing to its content of sodium bicarbonate the fluid is not stable, giving off carbon dioxide, whereby its reaction moves towards the alkaline. During cultivation, however, it is in a flask sealed with a rubber stopper and containing a gas mixture consisting of 2 to 6 % carbon dioxide in atmospheric air.

With this mixture an equilibrium is set up so that the pH remains constant. By varying the carbon dioxide content in the gas mixture the hydrogen-ion concentration can be so adjusted as to lie as near as possible to the optimum, which is $\text{pH} = 7.4$. The fluid contains phenol red as an indicator, and the pH is read by comparing the colour of the culture with a standard buffer solution of known pH to which the same quantity of phenol red has been added.

In my experiments the cultures were incubated at 35°. After a small quantity of minced embryonic mouse brain has been added to the fluid the flask is placed in the incubator for 24 hours for sterility control, whereafter it is stored at room temperature and in darkness until it is to be seeded. Seeding proceeds with 0.1 cc. of the material containing the virus. This gives a dilution of the proportions of 1:100 at each sub-culture. After seeding the flask is placed in the incubator, where it remains for 48 hours. The next passage can then be seeded, or the flask may be kept at room temperature in darkness for some days until it is required again. A single test showed no demonstrable decrease of the virus content after seven days' storage under these conditions.

The virus is cultivated in 23 passages. From the 2nd to the 16th passage subculturing was carried out simply by transferring 0.1 cc. of the fluid from one flask to another. From the 16th to the 23rd pas-

sage the fluid was centrifuged to ensure that no particles of tissue were carried from one passage to another.

The virus content of the supernatant fluid was titrated in the following manner: The culture was centrifuged. Of the supernatant fluid I made a dilution series, each step representing a dilution of 1:10 in saline with 1 % bacto-peptone. A fresh pipette was used for each dilution. The dilutions were injected into white mice weighing 16—18 g., either 0.05 cc. intracerebrally or 0.2 cc. intraperitoneally. The mice were observed for fourteen days.

After 23 passages the culture fluid reacted on the mice with typical pareses as far as the dilution 10^{-6} injected intracerebrally.

In preparing vaccine from the culture fluid I took advantage of the experience made at the State Serum Institute with the preparation of vaccine for foot-and-mouth disease as well as diphtheria vaccine (1, 4).

For the adsorption of the virus I employed a preparation of aluminiumhydroxide-gel with an aluminium content corresponding to 1.3 % Al_2O_3 . The method of preparation, which is of great importance to the adsorbability, is described by Schmit-Jensen, Schmidt & Hansen 1936 (4).*)

For making the vaccine the virus is grown in large flasks containing 100 cc. culture fluid. After the 48 hours' incubation the tissue particles are centrifuged off. The fluid is neutralized by the addition of hydrochloric acid, so that the reaction remains at $\text{pH} = 7.4$. This neutralization may be accompanied by a slight flocculation, which is removed by centrifuging.

The fluid is now intimately mixed with aluminiumhydroxide-gel in the proportions of 4 parts fluid + 1 part gel. The aluminiumhydroxide is then centrifuged to sedimentation, the supernatant fluid is poured off and replaced by a corresponding quantity of buffer saline with a pH of 7.38, in which the sediment is suspended.

On mouse-titrating the culture fluid, the supernatant fluid after adsorption, and the suspended sediment, it will be seen that practically the whole of the virus quantity has been adsorbed on to the aluminiumhydroxide. The supernatant fluid does not infect the mice in dilutions higher than 10^{-1} , indeed in most instances only the undiluted fluid produces infection, whereas the adsorbate has the same titre as the culture fluid. The results of one of these experiments are shown in table 2.

In order to ascertain the stability of the adsorption I made a test in which the sediment was washed three times by suspending it in buffer saline and then centrifuging. This test showed that the titer was the same before and after washing.

The virus charged aluminiumhydroxide suspension was now

*) The aluminiumhydroxide is prepared for the State Serum Institute by Dansk Svovlsyre og Superfosfatfabrik.

Table 2.
*Adsorption of virus to aluminiumhydroxide.
 The fluids are titrated intracerebrally on mice.*

Dilution	Culture fluid	Supernatant fluid after adsorption	Suspended sediment
undiluted		1:4	
10-1		0:4	
10-2		0:4	
10-3		0:4	
10-4	4:4		2:4
10-5	2:4		3:4
10-6	0:4		0:4
10-7	0:4		0:4
10-8	0:4		0:4

3:4 means 3 mice died from the 4 injected.

treated with formalin in various ways and then titrated on mice. The formalin employed is a commercial product containing about 40 % formaldehyde. The adsorbate was treated with various concentrations of formalin at a temperature of 25°. By this means it was found that the formalin concentration required to inactivate the virus in the course of 48 hours was between 0.05 and 0.1 %. This is shown in table 3.

Table 3.
Formalized aluminiumhydroxide adsorbate titrated intracerebrally on mice.

Dilution	0,05 % formaline 25° for 2 days	0,1 % formaline 25° for 2 days	0,05 % formaline 25° for 5 days
10-1	1:2	0:2	0:2
10-2	2:2	0:2	0:2
10-3	0:2	0:2	0:2
10-4	1:2	0:2	
10-5	0:2	0:2	
10-6	0:2	0:2	

For the immunization tests I used an adsorbate treated with 0.1 % formalin for three days at 25°. After this the formalin was not washed out; the mixture was kept in the refrigerator.

Whereas the infectivity of the adsorbate was thus abolished by the formalin treatment, it was found that the immunizing effect was still present.

These tests are described in greater detail in a following paper of Kauffmann and Frantzen.

Summary.

Jungeblut & Sanders' murine S. K. Poliomyelitis virus was grown in fluid tissue culture with embryonic mouse brain (after Jungeblut & Sanders).

A vaccine that is not infectious to mice but immunizes them against subsequent infection with the same virus was prepared from the supernatant fluid adsorbed to aluminiumhydroxide and treated with 0.1 % formalin at 25° for three days.

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MICE IMMUNIZATION TESTS WITH VARIOUS POLIOMYELITIS VACCINES AND SERA*)

By *F. Kauffmann and A. Frantzen.*

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The following experiments with mice are to be regarded merely as model experiments, made for the purpose of getting nearer to the problem of active immunization against human poliomyelitis.

We know quite well that the solution of this problem lies somewhere in the distant future, but we do hope that it will be attainable through the means of systematic research. For the present we have confined ourselves to experiments with mice, and so far we have worked only with two different murine virus strains, the one being Theiler's F. A. virus, and the other Jungeblut & Sanders' S. K. virus. In the following we shall call the latter virus »Columbia S. K.« to distinguish it from the »Yale S. K.« virus recently isolated by Melnick. In order to keep within the scope of this paper, however, we shall not discuss the question of the adaptation of human virus strains to rodents; we shall confine ourselves to a report on our immunization experiments.

Since it is possible according to Jungeblut & Sanders to cultivate Columbia S. K. virus in fluid medium with embryonic mouse brain, we have set ourselves the task of preparing, from a fluid tissue culture of this sort, an aluminiumhydroxide vaccine killed with formalin (see the foregoing paper of A. Frantzen).

In the first place, in a fairly large scale immunization test we injected mice of 16 to 18 grams intraperitoneally (= i. p.) with 0.2 cc. vaccine at intervals of three or four days. Seven days after the final injection the immunized mice and the equally old control mice were injected i. p. with living Columbia S. K. virus in decreasing doses.

Briefly stated, the result of that experiment was that the vaccinated mice proved to be well protected against subsequent infection by

*) Read before the International Congress of Microbiology in Copenhagen, July 1947.

Columbia S. K. virus. Whereas nearly all the control mice, which were infected with virus in a 1:100,000 dilution died, all the immunized mice survived, though they had received a dose a thousand times larger, that is to say, virus diluted to 1:100.

In a subsequent test the mice were immunized by being given the vaccine i. p., some four times, some twice and some once. Once was insufficient for protecting these mice; twice caused a distinct but not total immunity; four times produced an immunity just as good as in the first test with six injections. The results of tests with four immunizing injections are shown in table 1.

Table 1.
Active immunization of mice by a formalinized aluminiumhydroxide vaccine of Columbia S. K. virus (from fluid tissue culture).

S. K. virus i. p.	immunized mice (4 times i. p.)	control mice
10 ⁻¹	7:13	6:6
10 ⁻²	4:12	6:6
10 ⁻³	0:12	6:6
10 ⁻⁴	1:12	7:8
10 ⁻⁵	.	8:8
10 ⁻⁶	.	7:8
10 ⁻⁷	.	4:8

7:13 = from 13 mice 7 died.

. = not made.

Table 1 shows that it is possible actively to immunize mice with an aluminiumhydroxide vaccine. This being so, we now have a basis upon which we can prepare vaccines in a similar manner from other polio strains provided that we can cultivate these strains in fluid medium with embryonic brain, for instance human brain. When once we have the fluid culture, the preparation of an aluminiumhydroxide vaccine will not cause much difficulty.

In addition to this vaccine, for the sake of comparison we have also employed a formalin-brain vaccine of Columbia S. K. virus. To the virus-containing brain (diluted 1:10) we added 1 % formalin and then left it in the refrigerator for 8 days. It was then centrifuged and the brain washed twice with saline in order to remove the formalin. These formalin vaccines proved to be sterile and to protect mice in active immunization tests against intraperitoneal infection just as efficiently as the aluminiumhydroxide vaccine (see table 2).

These good results with a formalinized brain vaccine of Columbia S. K. virus are in conformity with those recently published by S. Gard (Nord. Med. 1947, 1399).

There being no detailed accounts of investigations into the immunological relations between Columbia S. K. virus and Theiler's F. A. virus, we have also prepared a formalinized brain vaccine from F. A. virus

Table 2.
Active immunization of mice by a formalinized brain vaccine of
Columbia S. K. virus.

S. K. virus i. p.	immunized mice (6 times i. p.)	control mice
10-1	0:6	.
10-2	2:6	.
10-3	0:6	.
10-4	1:6	3:4
10-5	0:6	4:4
10-6	0:6	3:4
10-7	0:6	1:4
10-8	.	0:4

3:4 = from 4 mice 3 died.

and immunized mice intraperitoneally. We then infected the mice i. p. with either F. A. virus (10-1) or Columbia S. K. virus. Likewise, mice immunized with Columbia S. K. virus i. p. were infected with S. K. or F. A. virus. The result was that Columbia S. K. vaccine afforded protection only against the homologous S. K. strain, but not against F. A. Conversely, F. A. vaccine protected only against the homologous F. A. strain and not against Columbia S. K. These results are in every way in conformity with those obtained by Ørskov and Kragh-Andersen on young mice infected per os (paper in the press).

We have supplemented these active immunization tests by employing both strains — Columbia S. K. and Theiler's F. A. — in the preparation of rabbit immune sera and by testing these sera in cross neutralization tests. S. K. serum neutralized S. K. virus, but not F. A. virus, and F. A. serum neutralized F. A. virus, but not S. K. virus. The results of these tests are shown in table 3.

Table 3.
Cross-neutralization tests with rabbit immunsera.

I. S. K. virus 10-1 + S. K. serum 1:10 equal parts 1 hour 37° C. 0.2 cc. i. p.	= 0:12
II. S. K. virus 10-1 + F. A. serum undil. equal parts	= 4:4
" " 10-2 + " " " "	= 3:4
" " 10-3 + " " " "	= 3:4
" " 10-4 + " " " "	= 4:4
1 hour 37° C. 0.2 cc. i. p.	
III. F. A. virus (4 brains) suspended in 12 cc. F. A. serum 1:10 1 hour 37° C. 0.5 cc. i. p.	= 0:20
IV. F. A. virus (4 brains) suspended in 12 cc. S. K. serum undil. 1 hour 37° C. 0.4 cc. i. p.	= 16*:19

0:12 = from 12 mice none died.

16*:19 = from 19 mice 14 died and 2 were paralysed only.

It will be seen clearly from table 3 that Columbia S. K. and Theiler's F. A. virus are 2 serologically quite different strains of murine poliomyelitis virus. Jungeblut has already demonstrated that his S. K. virus is also different from Theiler's G. D. VII virus and other spontaneous mouse-strains.

If we quite briefly want to touch upon the practical side of the problem — we mean the active immunization of man with poliomyelitis vaccine — we must say that we do not dispose of any experimental evidence arguing for the usefulness of a Columbia S. K. vaccine on man. We consider it doubtful that strains so intensively adapted to mice are of use for human purpose.

Summary.

The authors report on active immunization tests on mice with Columbia S. K. virus (Jungeblut & Sanders) and F. A. virus (Theiler).

When formalinized brain-vaccine was used, intraperitoneal treatment with S. K. vaccine provided protection only against an intraperitoneal S. K. infection, but not against an F. A. infection routed in the same way. Conversely, F. A. vaccine protected only against an F. A. infection, but not against an S. K. infection.

Working with a formalinized aluminiumhydroxide vaccine of Columbia S. K. virus cultivated in fluid tissue medium *a. m.* Jungeblut & Sanders, the authors obtained an immunity just as good as with a formalin-brain vaccine.

In neutralization tests with rabbit immune sera prepared from Columbia S. K. virus and F. A. virus, they found protection only against the homologous virus strain. Therefore F. A. virus (Theiler) and Columbia S. K. virus (Jungeblut & Sanders) must be regarded as two serologically different virus strains.

The authors discuss the problem of the active immunization of man against poliomyelitis, drawing attention particularly to formalinized aluminiumhydroxide vaccines, which must be made from suitable virus strains. It seems very doubtful that the Columbia S. K. virus is suitable for this purpose.

Our experiments are only to be regarded as model experiments, made for the purpose of getting nearer to the problem of active immunization.

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PHAGOCYTOSIS OF PATHOGENIC MICROBES ON SOLID CULTURE MEDIA BY AN AMEBA

By Kristian Ødegaard.

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Searching for antibiotic microorganisms in soil samples, an ameba was found, able to ingest pathogenic microbes growing on the ordinary solid culture media.

The ameba was found in a soil sample taken close to the beach in the inner part of the Oslo fiord. In its vegetative form the ameba has a diameter of about 12—18 μ . In the cystic form the diameter is 8—12 μ . The vegetative form may attain a length of 50—60 μ when it expands in one direction, what it tends to do in motile preparations. As well in the vegetative as in the cystic form the ameba has one nucleus only, relatively small and practically always situated centrally.

So far, one has succeeded growing the ameba with bacteria only, and it is easily grown with most of the microbes available in a bacteriological laboratory. It also grows on agar plates sown with killed bacteria.

Spreading a culture of yellow staphylococci on an agar or blood-agar plate and then placing the ameba on this culture, it will be observed, after an incubation period of 48 hours at room temperature, that the staphylococci have disappeared in an area around the ameba and is being replaced by a thin, greyish layer of amebas, chiefly the cystic form, mixed with some non-ingested staphylococci. The border-line between this layer and the intact microbes consists of a raised, slimy edge which marks the place where the vegetative forms of the ameba is fully active ingesting the bacteria. As these are being ingested, the amebas spread peripherally in all directions and has within three days, on an ordinary 2 per cent agar culture medium, spread about 1 cm. in all directions. If the medium is not too dry, the ameba will cover the entire plate (ordinary Petri dish). When the microbes are ingested, or if the culture medium gets too dry, cysts are formed.

However, some bacteria will survive in the area of phagocytosis.

This is easily demonstrated by making a new culture from this area. At the same time amebas will also be transferred to the new plate, and as soon as new bacteria grow up and thus supply nourishment for the amebas, these will immediately take the vegetative form and again start the phagocytosis.

Most Gram-positive cocci and intestinal Gram-negative rods are as easily ingested by the ameba as the staphylococci are, but Gram-positive rods are much more slowly phagocytosed.

The ameba grows and thrives well at room temperature. At 37 degrees it thrives poorly. To test the phagocytosis of microbes which do not grow at room temperature, the microbe is first incubated at 37 degrees for 24 or 48 hours. The ameba is then inoculated and the phagocytosis left to take place at room temperature.

Below a survey is given over the phagocytosis of different kinds of microbes. The intensity of the phagocytosis is indicated by +, ++, and +++. Ordinary lactose-bromthymolblue agar plates are used for Gram-negative rods. For most of the other microbes tested, blood-agar plates have been used, both with 2 per cent agar. The phagocytosis of tubercle bacilli was tested on Lowenstein's medium. The amebas were mixed with *Bact. Alcaligenes faecalis* and then transferred to the plates.

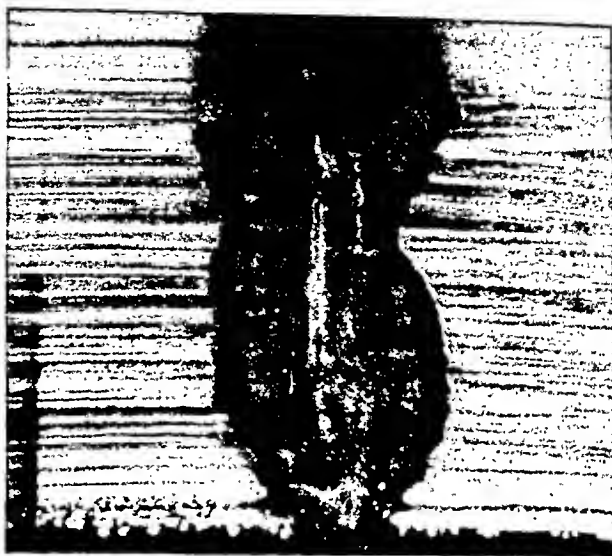
<i>Alcaligenes faecalis</i>	+++	<i>Staphylococcus aureus</i>	+++
<i>Escherichia coli</i>	+++	<i>Diplococcus pneumoniae</i> t. III	+++
Morgan I	+++	<i>Streptococcus hemolyt.</i>	++
<i>Salmonella typhi</i>	+++	<i>Bacillus subtilis</i>	+
<i>Salmonella paratyphi</i> B	+++	<i>Bacillus anthracis</i>	+
<i>Salmonella typhi murium</i> ...	+++	<i>Corynebact. diphtheriae</i>	+
<i>Shigella</i> III	+++	<i>Mycobact. tuberculosis</i>	+

As to the physical characteristics, the ameba did not survive a temperature of 60 degrees in half an hour. It also died when dehydrated, but freezing and thawing did not kill the ameba.

As to its classification, the ameba probably belongs to the tribe Vahlkampfia. This tribe consists of bacteria-ingesting amebas, which as a rule are small and in the cystic form have one nucleus only. They are widely distributed in the nature, partly free-living, partly in the intestine of different animals, such as, for instance, frogs and oysters. Handbooks of protozoology states that these amebas may be cultivated with bacteria on culture media, but it is not seen described in the literature how the amebas act upon pathogenic microbes.

The ameba was apathogenic to mice, $\frac{1}{2}$ cc. of a relatively dense suspension of the ameba (mixed with *Alcaligenes faecalis*) injected intraperitoneally failing to produce evidence of disease.

The ameba does apparently not produce any bacteriostatic or bactericidal agent. The fact that living microbes may be found on a plate where phagocytosis has taken place indicates this. The bacterio-



Phagocytosis of *Staphylococcus aureus* on blood agar, incubated for 72 hours at room temperature ($\times 2$).

(The amebas are streaked across the staphylococcal plate spread).

lysis seems to be connected with an endogenous process of the ameba.

One has not succeeded in an attempt of extracting bactericidal or bacteriostatic substances from the ameba, but these investigations will be continued.

Summary.

In a soil sample from the beach of the inner part of the Oslo fiord, the author has found an ameba which shows a great ability to phagocytose pathogenic microbes on the ordinarily employed solid agar media.

The ameba has one nucleus only, as well in the vegetative as in the cystic form, and has a diameter of 12—18 μ (vegetative) and 8—12 μ (cystic).

Further investigations will be carried out in order to try to find the bacteriolytic principle.

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STUDIES IN GRAM-NEGATIVE ANAEROBES. I. A HEMOPHILIC GRAM-NEGATIVE ROD

By *Sverre Dick Henriksen.*

(Received for publication October 2nd, 1947).

A great variety of gram-negative anaerobic rods have been described, and various, more or less successful attempts at classification have been made. In practical work with anaerobic infections, however, it is surprising how often one comes across strains that are difficult to classify or unclassifiable. It may be reasonable to suspect that only a fraction of the existing gram-negative anaerobic rods have been described so far, and there may consequently be reason to fear, that the wilderness, which this group represents to-day, may become increasingly confusing, until eventually some solid basis for a biological classification can be found.

Work has been in progress in this laboratory for a number of years in order to clarify the biological properties and classification of some microbes belonging to this group (Henriksen 1936, Thjøtta et al. 1939, Boe 1941, Boe and Thjøtta 1944, Lahelle and Thjøtta 1945, Lahelle 1947).

It is possible that the species, which have been studied so far, with a few exceptions, represent the fraction of the group that can be cultivated with the least difficulty, while microbes of this group with peculiar nutritional demands may yet be unknown.

In this and the following paper some such strains will be described. Due to the difficulties in cultivating these strains, the study of their biochemical activities is only fragmentary. Still their properties are sufficiently characteristic to make their recognition easy.

Description of strain.

Origin. This strain (844 L. F.) was isolated from a fatal case of pyemia, reported elsewhere (Henriksen 1947), caused by a mixed

anaerobic flora, including *B. fragilis*, *Fusobacterium*, anaerobic streptococci and another unclassifiable strain of gram-negative rods. There was no reliable evidence to decide which of these microbes were of primary pathogenic importance.

Isolation. No colonies of this strain were found in the primary culture. As a number of fusiform rods were seen in films of the pus, attempts were made to isolate these by means of selective bacteriostasis in tubes of broth containing a piece of fresh potato tissue and gentian violet. By spreading the 4th and the 5th subculture in such tubes on blood agar, colonies of a typical strain of *Fusobacterium* and of strain 844 L. F. were found, after anaerobic incubation for many days. The latter colonies were tiny, just visible (about 1/4 mm), flat and transparent.

Morphological properties. Very thin fusiform gram-negative rods, measuring about $3-6 \text{ my} \times \frac{1}{2}-\frac{3}{4} \text{ m}$, straight or slightly curved, with thin pointed ends, and mostly of fairly uniform dimensions. The appearance in solid and liquid media was the same. The most striking peculiarity was a distinct, spherical or slightly oval swelling at the middle of many of the rods, measuring $1\frac{1}{2}$ to 2 my. The swellings were evenly, often strongly stained, and were not found in a free state. The rods were gram-negative with a low affinity to the counter stain. A strong stain such as carbol fuchsin had to be used to get clear pictures. (Photo no. 1).

No motility could be detected.

Cultural properties.

Cultural properties. On blood agar plates colonies could seldom be seen after the first three days' incubation. After 4 to 5 days or later they appeared as minute dust particles on the surface. In the course of 10 days to 3 weeks they increased up to a diameter of $\frac{1}{2}$ mm or occasionally 1 mm. In young cultures they were round, flat, slightly irregular. With increasing age the irregularities increase and after 14 days to 3 weeks the colonies have an irregular dentate or lobate margin. Growth on blood agar is uncertain. On some plates growth is fairly good, while on others it may fail altogether. On such plates one may sometimes find a small group of typical colonies arranged as satellites within a few millimeters of contaminant colonies. Even on plates which gave fairly good growth, it was denser and with larger colonies round contaminant colonies. Practical use was made of this preference for symbiotic growth to ensure growth on the plates, a *Staphylococcus* being used as the growth-promoting organism. (Photos nos. 2-4).

No growth on solid media without blood.

No growth was obtained in broth, peptone water or semisolid brain heart medium, while in tubes of broth containing a piece of fresh potato tissue a very faint turbidity appeared after 48 to 72 hours.

sometimes starting as a fine cloud round the potato. Neither gas nor odor nor indol were produced.

Growth was also obtained in broth containing 10 % blood or blood and a piece of potato. In broth containing the V-factor of Thjøtta and Avery, in the form of yeast extract, or the V and the X-factor (autoclaved blood extract) a very slight sediment of growth was obtained, while no trace of growth could be seen in tubes of broth with the X-factor alone or without either factor.

Addition of 1 or 2 % of glucose to liquid media did not improve growth, nor could any significant acid-production be found (pH 6.48 as against 6.75 in a tube without glucose).

No growth occurred in aerobic conditions or in a partial CO₂ atmosphere.

Pathogenicity. Organisms from potato broth or from blood agar plates were inoculated intra-abdominally into mice. No reaction occurred.

The strain has been under cultivation for 1½ years and has remained constant throughout this period.

Discussion.

No reference has been found to any organism of these properties. It is evident that this strain must be considered a hemophile, in so far as it requires the V-factor, although not the X-factor. At present no hemophilic anaerobic gram-negative rod is included in the genus *Hemophilus*, nor in *Bacteroides*.

Still it is not surprising that such microbes should exist. It is very likely that microbes showing the same deficiencies in their enzymatic equipment may develop from widely separated progenitors. There is e. g. no reason to believe that all the different anaerobes known to day have any common phylogenetic origin. Nor is there necessarily any reason to believe that the hemophilic property signifies a common phylogenetic origin. Although such properties may be useful in separating microbes into groups for practical purposes, a classification based on such properties alone may perhaps lead to a false conception. In this connection it may be mentioned that two strains of grampositive rods with hemophilic properties, tentatively classified as *Corynebacteria*, were recently described from this laboratory (Svendson et. al. 1947).

The classification of our strain may consequently be considered doubtful. According to its growth characteristics it might be classified either as a *Hemophilus* or as a *Bacteroides*, and if its morphological properties are considered, classification as a *Fusobacterium* might seem reasonable, although it shows none of the more striking biochemical activities of the genus.

It is felt that final classification should be postponed until more strains can be found and studied. Temporarily it might be convenient to consider it a member of genus *Bacteroides*, which already is hetero-

geneous enough to admit some new members. It is possible that this strain may be found to represent a new species. As strains of this type are difficult to isolate and may easily be missed in the cultures, it is felt that the chance to isolate similar strains is a rather uncertain possibility, greatly dependent on luck. Therefore, it is thought to be justifiable to describe this single strain.

Summary.

A strain of thin, fusiform gram-negative anaerobic rods was isolated from a case of anaerobic infection. It was characterized by the production of spherical swellings at the middle of the rods, by an absolute requirement for the V-factor and a preference for symbiotic growth with other organisms. It is suggested that the strain should be considered as belonging to the genus *Bacteroides*, until further evidence can be obtained.

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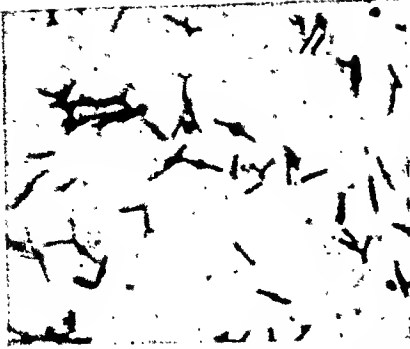


Photo no. 1.

Microbes from 72 hours' potato
broth. Stained with carbol fuchsin.
× 1850.



Photo no. 2.

Colonies on blood agar. 33 days.
× 16.



Photo no. 3.

Colonies growing as satellites
around contaminant. Blood agar,
13 days. × 16.



Photo no. 4.

Dense growth of small satellite
colonies round contaminant.
Blood agar, 11 days. × 16.

STUDIES IN GRAM-NEGATIVE ANAEROBES. II. GRAM-NEGATIVE ANAEROBIC RODS WITH SPREADING COLONIES

By *Sverre Dick Henriksen.*

(Received for publication October 2nd, 1947.)

A number of aerobic and anaerobic species produce spreading colonies on solid media, but apparently this type of colony has not been found in any of the gram-negative, non-spore-forming anaerobic rods.

Some strains isolated in this laboratory have shown this property and also other peculiarities. In many respects these strains were similar, while they differed in others. Consequently they may conveniently be described together, with the reservation that they may not necessarily belong to the same species.

Description of strains.

Origin. One strain (1424) was isolated from the genital tract of a woman suffering from endometritis. The second (K. A. 93) was isolated from the pus of a feline pulmonary abscess, while the third (A. J.) was isolated from the pus of a perineal abscess. In all cases several other anaerobic organisms were isolated.

Isolation. Strain 1424 was isolated from the primary blood agar plate, by fishing some tiny colonies after several days' incubation. Strain K. A. 93 was only obtained in pure culture after about 6 weeks. At first it grew only in mixed colonies with several other organisms, and a number of replatings were necessary before this mixture could be separated into the individual components.

Strain A. J. was isolated from the primary culture after 8 days' incubation. No isolated colonies of this strain were found on the plate, but subculture of a thin spreading film which surrounded a group of different colonies yielded a pure culture of this strain. In all cases

isolation would have been impossible unless the primary and secondary plates had been incubated for a number of days.

Morphology. All strains were small, slender gram-negative rods. All strains also produced a number of very small rods and tiny gram-negative particles. One strain was rather homogeneous, producing mainly short and medium, slender, straight or slightly curved rods (photo nr. 1). The other strains were more pleomorphic with a predominance of tiny particles, and one of them (A. J.) also usually produced some larger, swollen rods and irregular structures (photos nos. 2 and 3). All strain were gram-negative and took the counter stain poorly. A strong stain like carbol fuchsin had to be used to differentiate all the elements.

One strain (K. A. 93) showed an unusually active motility, both from blood agar surfaces and from liquid media. Often the rods moved so fast that it was difficult to get a view of them before they had crossed the entire field. Others rotated rapidly or showed pendulating movement back and forth.

In spite of numerous attempts no motility could be detected in the two other strains at any stage of growth.

Cultural properties. All strains grow very slowly on blood agar. After 48 hours the colonies are barely visible, or visible only with magnifying glass. Young colonies were of two types, either slightly raised, flat, smooth or slightly irregular, or they appeared as depressions in the surface instead of raised colonies. These depressions were shallow, circular or slightly irregular, and they varied in size from about 0.2 mm. to about 1 mm. (photos nos. 4 and 5). Such depressions were seen in cultures of all strains, but with varying frequency. In strain A. J. the depressions were the rule, whereas raised colonies were rare exceptions. In strain K. A. 93 some cultures contained mainly depressions, others mainly small irregular flat colonies (photo no. 6). Strain 1424 usually started growth as tiny, smooth colonies, and depressions were only found in occasional cultures. On continued incubation the depressions gradually were filled with bacterial growth, and became surrounded by a low, narrow wall with a granulated surface (photo no. 7). At the same time many colonies were surrounded by a delicate halo of growth, at first visible only as a slight dullness of the agar surface. Later this first halo became somewhat thicker with a finely granulated surface, while a new thin halo was formed beyond it. Finally, after 3 to 4 weeks in the incubator, a series of concentric zones of growth could be seen, resembling the zones formed round colonies of *Protens vulgaris*, but on a greatly reduced scale (photos nos. 8—10). Growth never was heavy, and only a small quantity of microbes could be scraped off even old cultures. This concentric spreading growth was especially marked in strain A. J., while in the other strains it was more irregular and sometimes did not occur to any great extent. Growth seemed to continue for at least 4 weeks.

After 2 weeks or more a gradual digestion of the blood under the growth appeared, and sometimes clear, transparent zones might be formed.

On ascites agar growth is much poorer than on blood agar, and subculture on new ascites agar plates usually fails. On lactose agar a meager growth may be obtained with two of the strains (A. J. and 1424), whereas strain K. A. 93 failed to grow. The two former strains might also produce a feeble growth on a second, and occasionally a third, lactose agar plate, after which growth failed entirely. It is possible that some growth stimulating substance may have been carried over from the blood agar plate with the inoculum.

For a considerable time it was believed that these strains were unable to grow in liquid media, as no signs of growth could be seen in any such medium, and microscopic examination of the cultures gave very uncertain results. However, it was later found that these strains multiply in certain rich media, although the multiplication apparently is too slight to produce visible turbidity or sediment.

In tubes of broth containing 10 % blood or blood and a piece of potato, no turbidity stronger than in uninoculated control tubes could be seen (strain K. A. 93 possibly produced a just perceptible turbidity in certain young cultures). On microscopic examination of these cultures, a moderate number of very active motile rods could be seen in strain K. A. 93, while cultures of the other two strain only contained a very small number of nonmotile rods, which might very well only represent the inoculum. Further, a number of tiny particles were seen, but similar particles were also found in uninoculated tubes, and no clear-cut differentiation could be made. However, subculture of these tubes on blood agar after 24 hours to 30 days gave growth of a considerable number of typical colonies. Cultivation in a series of blood broth tubes was attempted, and subculture of the 6th transfer in blood broth on blood agar plates gave just as good, or better, growth than from the first tube. Thus there is no doubt that some growth must occur in such media, and that it can be brought to continue, probably for an unlimited period of time.

Similar results were obtained with broth, or ascites broth, containing a sterile piece of animal tissue (guinea pig kidney or liver). On the other hand no growth occurred in peptone water, plain broth, broth with a piece of potato or semisolid brain heart medium.

Neither gas nor odor were produced in any fluid medium.

No growth was obtained under aerobic conditions.

Pathogenicity. Mice showed no reaction after intraabdominal injection of a saline suspension of microbes from blood agar plates.

Discussion.

The strains described above do not seem to conform with any species described so far. In their morphological and cultural properties

all strains are very similar, but the fact that one strain was found to be motile, whereas no motility could be demonstrated in the others, may suggest a difference. The possibility should be considered, however, that the failure to demonstrate motility in two of the strains may have been due to some technical difficulty rather than to lack of motility. It is well known that motility can only be demonstrated in some anaerobes if exposure to oxygen is prevented or reduced to a minimum. It is possible that some such difficulty may be the reason in this case also. Otherwise these strains did not seem to be very sensitive to oxygen, and successful subcultures could be obtained even after the plates had been exposed to air for several hours.

The strains were characterized by a number of peculiarities. The most striking of these, perhaps, is the tendency to start growth on solid media in the form of depressions rather than raised colonies. The cause of this phenomenon is unknown. It does not appear to be due to digestion of the agar, since no progressive digestion occurs even after very long incubation.

A second peculiarity is the tendency to produce a continuous, spreading growth, with concentric zones of spread in a similar manner as in *Proteus*. Spreading is not very uncommon, and is known in a number of aerobic and anaerobic species (*Proteus*, *Knrthia*, *Clostridium*), but in these cases it is always accompanied by active motility of the individual organisms. Whether motility is actually the cause of swarming may perhaps not be quite settled. The fact that many strongly motile organisms do not spread on solid media, may suggest some additional mechanism in those that do spread. Still, spreading in non-motile organisms would be unusual.

Finally the slow growth, feeble growth energy and failure to grow on simple media are very characteristic of these strains. It is not known which growth substances the strains demand, but it appears to be some substance, or substances, contained in blood and animal tissues, but not in plant tissue.

In spite of their poor and slow growth, the organisms seem to be rather hardy, and keep alive for a considerable time — at least 30 days in culture at 37 C.

Final classification of these organisms should probably not be attempted until more strains have been studied and a more detailed knowledge of their properties has been obtained.

Two possibilities of classification suggest themselves. One is to consider them members of Genus *Bacteroides*. With regards to strain K. A. 93, at least, classification as an anaerobic vibrio would be another possibility. The fact that these rods were very actively motile, and that they often appear to be somewhat curved may be mentioned in support of this. Previous authors have frequently found motile anaerobic vibrios in anaerobic infections (Smith 1928, 1930a, 1930b), but no detailed description of the properties of such vibrios has been found.

Summary.

Three strains of gram-negative anaerobic rods, isolated from cases of mixed anaerobic infection, are described.

They were characterized by a very slow and meager growth, by a tendency of young cultures to produce shallow depressions in the blood agar surface, instead of colonies, and by a continuous, spreading growth, in the form of concentric zones. Motility could only be demonstrated in one strain.

They seemed to be very inactive biochemically and to require rich media. Classification is discussed.

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Photo no. 1.

Strain K. A. 93. From 7 days' blood agar culture. Stained with carbol fuchsin. $\times 1500$.

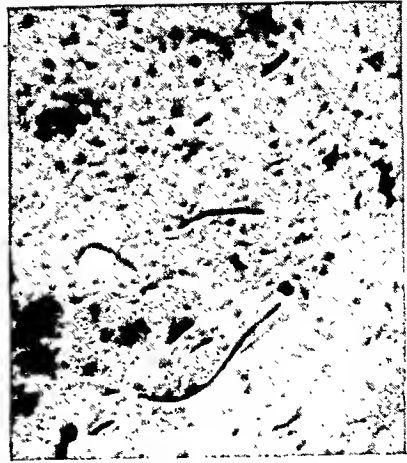


Photo no. 2.

Strain 1424. From 8 days' blood agar culture. Stained with carbol fuchsin. $\times 1850$.

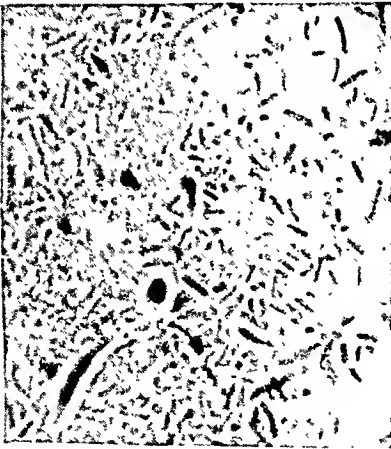


Photo no. 3.

Strain A. J. From 6 days' blood agar culture. Stained with carbol fuchsin. $\times 1850$.



Photo no. 4.

Strain A. J. Blood agar, 4 days. Shallow depression and tiny, raised colony. Photographs unfortunately give the false impression that the depressions are convex. $\times 17$.



Photo no. 5.

Strain K. A. 93. Blood agar, 4 days. Small crater-shaped colonies, consisting of central depression, surrounded by wall of growth. $\times 16$.

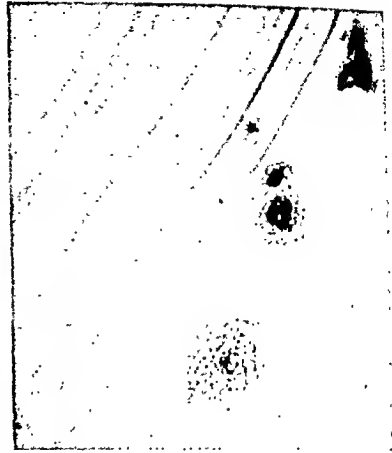


Photo no. 6.

Strain K. A. 93. Blood agar, 3 days. Flat, irregular colonies. $\times 16$.

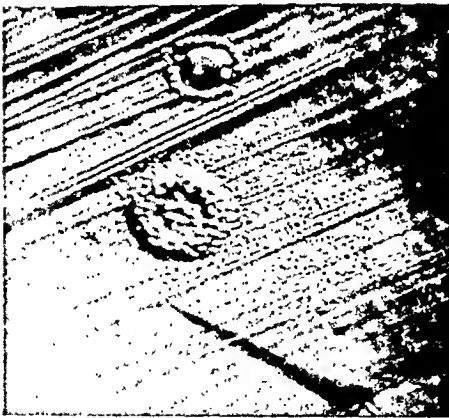


Photo no. 7.

Strain A. J. Blood agar, 8 days. Depression which is in the process of being filled out with growth, surrounded by wall. The original photograph shows that the agar surface surrounding the colony is finely granulated, due to a thin film of growth. $\times 14$.

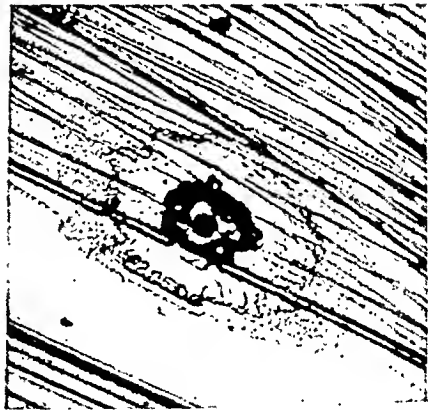


Photo no. 8.

Strain K. A. 93. Blood agar, 21 days. Colony surrounded by two concentric zones of growth. $\times 16$.

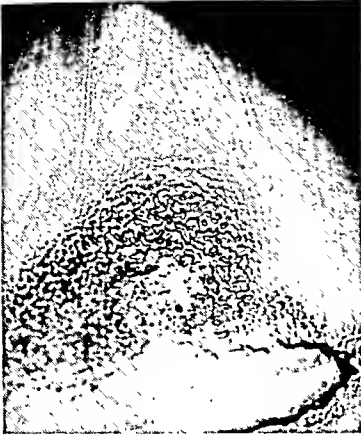


Photo no. 9.

Strain 1424. Blood agar 22 days.
 Raised, smooth plateau, surrounded
 by a lower, granulated zone, and a
 very thin film, $\times 17$.

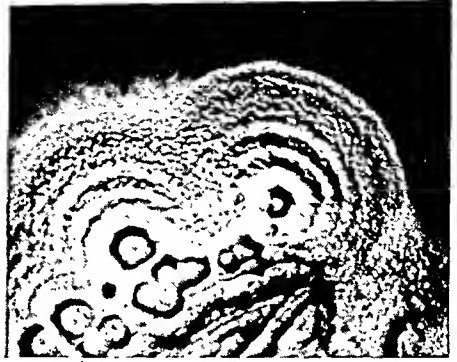


Photo no. 10.

Strain A. J. Blood agar, 21 days.
 7 concentric zones of growth. $\times 15$.

SOME INVESTIGATIONS INTO THE PENICILLIN SENSITIVITY OF HUMAN-PATHOGENIC ACTINOMYCETES

AND SOME COMMENTS ON PENICILLIN TREATMENT OF ACTINOMYCOSIS

By *Per Holm*.

(Received for publication October 17th, 1947).

According to reports from the last 3—4 years penicillin has been used for treatment of quite a number of cases of human actinomycosis: *M. E. Florey & H. W. Florey* (1943), *Keefer et al.* (1943), *Herrell & Nichols* (1943), *Vaughan Hudson* (1943), *Lyons* (1943), *Herrell* (1944), *Christie & Garrod* (1944), *Wollgast* (1944), *Herrell, Nichols & Heilman* (1944), *Meleney* (1944 b), *Herrell & Kennedy* (1944), *Roberts, Tubbs & Bates* (1945), *Walker & Hamilton* (1945), *MacGregor* (1945), *McCrea, Steven & Williams* (1945), *Nacnab* (1945), *Hendrickson & Lehman* (1945), *Dobson & Cutting* (1945), *Hamilton & Kirkpatrick* (1945), *Herrell* (1946), *Vaughan Hudson* (1946), *Abrahams & Miller* (1946), *Meleney* (1946), *Piper* (1946), and *Lachmann* (1946).

It appears from the reports that in some cases penicillin seems to have had a curative effect on the disease, whereas in other cases it has been without any effect at all.

With regard to the treatment of patients with actinomycosis it will be of interest to find, why the penicillin does not influence some patients. By reading the various authors' cases it will be seen at once that some of the patients have received very little penicillin, while others have received large doses, that some have been treated for a short time, others during a longer period. There is no doubt that the duration of the treatment and the size of the doses must be important.

To obtain favourable results when treating an infectious disease with an antibiotic it is, however, a necessity that the agent of this illness is sensitive to the substance in question. Consequently, it is

of great interest to determine whether ray fungi can be influenced by penicillin. Already more reports on such investigations have been published. It is evident from the following that these sensitivity determinations have been performed with vast differences in technique and results.

Abraham et al. (1941 (the Oxford team) have examined a strain of »A. bovis (hominis)« by cultivation in deep glucose agar shake culture with penicillin; they state that the strain examined had the same degree of sensitivity as *Staphylococcus aureus*.

Fisher (1943) has examined 2 strains of »*Actinomyces bovis*«. He writes nothing about the characteristics and origin of the strains concerned, nor does he write anything about the way in which the experiment has been conducted; it is recorded in his work that he found the strains to be 8–10 times as resistant as *Staphylococci*.

Garrod (1944) has determined the penicillin sensitivity of 6 strains of *Actinomyces* originating from patients treated by *MacGregor* and by *Roberts, Tubbs & Bates*. About one of these ray fungi, cultivated from a patient with actinomycosis of the lung, it is reported that it »was.... an atypical strain«. *Garrod* determined the sensitivity of *Actinomyces* by cultivation in a series of broth tubes containing decreasing concentrations of penicillin anaerobically incubated for 10 days, an identical series being inoculated with the Oxford H strain of *Staph. aureus* as a control. His experiment shows that some of the strains of *Actinomyces* had the same degree of sensitivity as the standard *Staphylococcus*, others being 8–16 times as resistant.

Keeney et al. (1944) have examined »an anaërobic actinomycete, a cause of human actinomycosis«. They write the following about the investigations: »Tubes of thioglycollate media containing 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 and 10.0 Oxford units of penicillin, respectively, were inoculated with 0.1 cc. of the granules and media of a 4 weeks old culture of *Actinomyces bovis*, and incubated at 37° C. for 3 weeks. Freshly prepared penicillin was re-added to the tubes every 48 hours so that the original concentrations of penicillin could be maintained throughout the entire period of 3 weeks. The growth in the penicillin treated cultures was compared with the growth in 2 control cultures.

At the conclusion of these experiments the inocula were removed as completely as possible from those tubes in which there had been obvious or complete inhibition of growth and transferred to tubes of unmedicated thioglycollate media. These tubes with the transplanted inocula were incubated at 37° C. for 4 weeks and noted for growth. If the granules failed to increase in size or multiply during this time of incubation, we assumed that they had been killed as well as inhibited by the previous exposure to penicillin«. They found that their strain of *Actinomyces* had been killed by concentration of 0.01 Oxford unit of penicillin per millilitre medium.

Dobson & Cutting (1945) have investigated the penicillin sensitivity of 3 strains of *Actinomyces*. All 3 of them are most likely to be old laboratory strains; at least one of them has nothing to do with actinomycosis, as it is evident from their table 2 that the strain has been isolated from the air. For 7 days they cultivated the strains in thioglycollate medium, containing varying concentrations of penicillin. From a table it is seen that their ray fungi grew in broth, containing 1½ units of penicillin per millilitre, but that they were somewhat inhibited in growth by this concentration.

Concerning experiences from the Mayo Clinic, *Herrcll* (1946) writes in his book: »Penicillin and other Antibiotic Agents«, the following: »Nearly all strains of *Actinomyces bovis* have been found to be fairly sensitive to the action of penicillin«.

Abrahams & Miller (1946) cultivated from 2 patients 2 strains of Actinomyces showing »individual morphological and cultural differences«. They determined the influence of the penicillin on the 2 strains in the following way: »One—tenth millilitre of a 96-hour broth culture of each strain was inoculated into tubes of glucose (1.0 per cent) nutrient agar containing varying concentrations of.... penicillin.... The inoculum was thoroughly mixed with the agar and the culture was incubated at 37° C. Density readings were taken after 96 hours«. Both strains were totally inhibited by 0.2 units of penicillin per millilitre medium, one of them also by 0.1 units.

Author's Investigations.

In Denmark the bacteriological examination of specimens of pus, expectoration etc. for Actinomyces, has for a number of years been centralized at the State Serum Institute, where about 350 samples are now examined yearly for that microbe. In connection with the routine diagnostic work, a series of different (not yet published) examinations on the bacteriology of actinomycosis has been performed. Accordingly, it seemed appropriate also to submit to closer examination, the problem of the penicillin sensitivity of human-pathogenic Actinomyces, especially as the continuous supply of samples gave easy access to freshly isolated strains.

On Selection and Pure Cultivation of the Actinomyces-Strains.

The micro-organisms of the Actinomyces-group can be divided into numerous species, which are to be found widely spread. They are met with in soil, in the atmosphere, in water, on the mucous membranes of the warm-blooded animals, for example in the oral cavity of man, and most likely also on the skin. Many of the ray fungi which are described as originating from the disease products in man, are actually contaminations, which have either been mixed into the pus during the taking, or into the media during the inoculation and cultivation.

To draw conclusions from investigations into the penicillin sensitivity of the Actinomyces regarding the possibilities of treating patients suffering from actinomycosis with this substance, it is of vital importance that those strains examined have actually been the cause of disease.

Is it at all possible to decide whether a microbe is pathogenic, and how?

According to Löffler, (1887) as well as to Bulloch, (1938) Hentle (1840) is said to be the first one to ponder over that problem. In Hentle's time, the causes of the infectious diseases were unknown, but it was supposed that they were brought about by some agents, called contagia. The thought occurred to Hentle that these agents must be of organic nature, living, in possession of individual life, and that in relation to the diseased organism they must be parasites. He presumed that these contagia must be »bewegliche Thierchen oder dentliche Pflanzen«, he knew one contagium, namely the mould detected by

Bassi which is the cause of Muscardine, a disease of silk-worms; he had searched for contagia in the disease products in man, but did not find them, and was of the opinion that it would be hard to find them. He would, however, acknowledge a contagium as being the cause of an infectious disease, 1) if it could constantly be found during it, 2) if it could be isolated, and 3) if by means of the isolated contagium it was possible to produce the same illness again experimentally.

Those are *Henle's* conditions or as *Löffler* (1887) called them: »die drei Postulate der strengen Logik *Henle's*«. Sometimes, the same conditions are mentioned as *Koch's* postulates (see *Fildes & McIntosh* (1920), *Bullock* (1938)), which is probably partly due to the fact that in his work from 1884: »Die Ätiologi der Tuberkulose« *Koch* had advanced exactly those 3 conditions to acknowledge the tubercle bacillus found and cultivated by him as the cause of tuberculosis.

Have all those 3 conditions now been fulfilled as far as the actinomycosis is concerned? By no means. True enough, a characteristic microbe: *Actinomyces*, has permanently been found by microscopy. Thus condition no. 1 must be said to have been fulfilled.

What then about the second condition: the isolation of the microbe? Here it must be said that quite a number of research workers who have investigated the etiology of the actinomycosis, have undoubtedly isolated the pathogenic *Actinomyces*, but this is far from being valid for all of them; a great many authors have used such methods for pure cultivation, that they have been unable to decide whether a possibly cultivated *Actinomyces* was a contamination, or whether it was the microbe occurring in the pus.

Condition no. 3 is but seldom fulfilled; only exceptionally, has it been possible to develop experimental actinomycosis in animals, not even by *Actinomyces* strains cultivated from patients.

Supposing one had to follow *Henle's* or *Koch's* strict rules, one might actually think that the main part of the *Actinomyceles*, up to now being cultivated from human beings with actinomycosis, are non-pathogenic, and in this way have nothing to do with the disease of the ray fungi. This point of view can, however, not be true even if there is no doubt that some of them are contaminations. Other human-pathogenic microbes also are known to be non-pathogenic to animals, and we, consequently, have to find a different way in which to draw nearer to the solution of the problem. Again we turn to *Robert Koch* and now find that in an earlier work (from 1881) he has given a different definition of what is understood by a pathogenic microbe. In his paper: »Zur Untersuchung von pathogenen Organismen« he writes the following: »Sobald also Bakterien, und dasselbe gilt ganz ebenso von anderen Mikroorganismen, im Innern der Organe ... oder im Gewebe selbst ... gefunden werden ... dann müssen solche Mikroorganismen als pathogen angesehen werden, mindestens müssen sie verdächtig erscheinen und zur weiteren Untersuchung und Aufklärung

des Befundes auffordern.« Adhering to that definition, it will at all events now and then be possible to find a strain of *Actinomyces* of which can be said that in all probability, amounting almost to certainty, it is pathogenic. Being patient one may by degrees collect a series of strains, to be designated as pathogenic.

As initial material when collecting such strains, only pus taken from closed lesions can be used (i. e. abscesses or empyema not yet incised), otherwise one cannot be certain that possibly detected microbes do originate from »the interior of the organs or from the tissues.« The samples must be taken by puncture with a sterile syringe or after incision through previously disinfected skin, (not through mucous membranes which can only with great difficulty be sterilized efficiently). Furthermore, the very cultivation from the samples must be carried out in such a way as to show from the result of the bacteriological examination, whether an *Actinomyces*, originating from the pus, has been detected, or whether it is a contamination, grown on the media. As will be seen, great demands are made on pure cultivation.

Regarding the present investigations, it was particularly fortunate that I had previously worked out a method for cultivation of ray fungi from pus. Later on, a report will be published on these experiments, here is mentioned only that blood proved to contain substances which had a growth-increasing effect on all human pathogenic *Actinomyces*. Carbon dioxide also had a favourable influence on the growth, and its presence was necessary to obtain growth of some strains. Moreover, it appeared from the experiments, that the *Actinomyces* were able to grow on the surface of solid medium (blood-agar), when the cultivation took place in jars under anaërobic conditions. In practice, examination of a sample from a patient suffering from actinomycosis is performed in the following way: By means of a Pasteur pipette 2—3—4 granules (a little pus, if granules are not present) are procured and placed on a sterile slide with some broth. The granules are then crushed with another slide as carefully as possible. From the very thin suspension made this way, 4 blood-agar-plates are inoculated (nutrient agar with 10 % of sterile horse-blood). 2 of those plates are cultivated aërobically in the usual way in an incubator, the 2 others are placed in a Zeissler-jar (1928), from which the air has been exhausted by a Pfeiffer's oil-pump. A vacuum having been obtained in the jar, about 5 % of carbon dioxide is generated by means of sodium bicarbonate and sulphuric acid, placed in the jar beforehand (principle: *Marlin Kristensen*, 1928). The inoculated plates are incubated for 5 days at 37° C.

The method is good. If many filaments have been detected on microscopic examination of the pus there will, as a rule, also be growth of many colonies of *Actinomyces* on both plates cultivated anaërobically and of almost the same number on each of them. When this agree-

ment is present between microscopy and cultivation, one is entitled to conclude that the cultivated Actinomyces was found in the pus, and in such cases where many colonies of Actinomyces have been developed, a pure colony may be transplanted, and this culture incorporated in the collection of the human pathogenic strains. On the other hand, one should not keep the strain, if only a few colonies of Actinomyces are found on the plates, and especially if these appear on one plate only. In all probability, a pathogenic strain may no doubt be seen in a number of these cases, but there is no definite basis for it. In addition, the method of cultivation mentioned above is not only qualified for scientific use, but it has also proved immensely valuable for the routine diagnostic examinations. Nearly always it yields growth of Actinomyces, this microbe having been detected by microscopy. In some cases growth has been obtained, when microscopy had not led to any positive results. The method fails, however, if besides Actinomyces the pus also contains proteus bacilli or other swarming microbes.

Record of the Pure Actinomycetes and Their Bacteriology.

The strains for the examination mentioned in the following have been collected according to the above indications, and they are entered in table 1. On each strain information is given as to whether the sample of pus has been taken from an abscess or from an empyema, how the pus has been taken, whether disinfection has been carried out before the taking, and the result of the bacteriological examination, especially reporting how many colonies of Actinomyces were found at the primary cultivation of the plates inoculated with pus or granules. In table 2 a few clinical notes are given of the patients from whom the strains have been cultivated. Here, the localization of the lesion is noted, as well as for how long the patient had been ill when the ~~sample of pus was examined. Finally, the answers to 2 questions, put~~ to the physicians sending in the samples, are given. The strains have been isolated from samples sent to the institute during 2 periods, from 8/7 to 29/12—40, and from 3/8—45 to 16/5—46, respectively. All the strains from 1940 were dried immediately after pure cultivation by Flosdorf-Mudd's method (1935) and were kept in this state directly up to the time of their examination. The strains from the last period were sown in semi-fluid agar, and thereupon kept at room temperature, until the experiment was commenced.

All the ray fungi are isolated on the plates incubated anaërobically, none are able to grow aërobically on the surface of nutrient agar, they are Gram-positive, and all of them grow in mycelium. This last fact has been demonstrated by direct agar microscopy (Ørskov, 1923) after inoculation and anaërobic cultivation on the surface of ascitic agar.

As will be seen from the tables 1 and 2, the strains have been classified into 4 groups. The first group comprises (strains 1—20) ray

Table I.

Serial Number	Number of Journal	The Sample Taken:			Disinfection in advance by means of	Examination of the pus		
		from	by	through		Presence of Fibrules	Microscopy of granules (pus):	Cultivation: Number of colonies per primary spreading-plate at the anaerobic cultivation.
1	818/40—41	Abscess	Puncture with sterile syringe	Skin	Tincture of Iodine	—	— Mycelia - Filaments	Approximately fifty
2	1061/40—41	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	A few hundred
3	1311/40—41	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
4	1435/40—41	Abscess	Incision	Skin	Propyl alcohol	+	— Mycelia - Filaments	Approximately fifty
5	2129/40—41	Abscess	Incision	Skin	Propyl alcohol	+	+ Actinomycotic filaments	About a hundred
6	1651/40—41	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
7	1919/40—41	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	A great many
8	2481/40—41	Abscess	Incision	Skin	Ether and alcohol	+	+ Actinomycotic filaments	About a hundred
9	101/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
10	169/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	More than a hundred
11	170/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
12	175/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	A hundred at least
13	186/45—46	Pleural cavity	Puncture with sterile syringe	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
14	195/45—46	Pleural cavity	Puncture with sterile syringe	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
15	223/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	25—30

16	231/45—46	Abscess	Puncture with sterile syringe	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
17	260/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Some hundreds
18	297/45—46	Abscess	Incision	Skin	Alcohol	+	+ Actinomycotic filaments	A few hundred
19	39/46—47	Abscess	Incision	Skin	Tincture of Iodine	—	+ a few Actinomycotic filaments	40—50
20	54/46—47	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
21	1406/40—41	Abscess	Operation	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Numerous
22	1452/40—41	Abscess	Incision	Skin	Propyl alcohol	+	+ Actinomycotic filaments	Several hundreds
23	161/45—46	Abscess	Puncture with sterile syringe	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	A great many
24	265/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Some hundreds
25	268/45—46 _A	Abscess				+	+ Actinomycotic filaments	About a hundred
26	311/45—46	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
27	8/46—47	Abscess	Incision	Skin	Tincture of Iodine	+	+ Actinomycotic filaments	Several hundreds
28	227/45—46 _n	Abscess	Puncture with sterile syringe	Skin	Tincture of Iodine	+	+ slender Gram-positive bacilli and short filaments	Several hundreds
29	253/45—46	Pleural cavity	Puncture with sterile syringe	Skin	Tincture of Iodine	—	+ slender Gram-positive bacilli and short filaments	A few hundred
30	227/45—46 _A	»Associate« See above notes concerning strain 28						Several hundreds
31	268/45—46 _B	»Associate« See notes above concerning strain 25						Several hundreds

Some notes on the taking and examination of the pus samples, having been used as initial material at the pure cultivation of the strains of Actinomycetes.

Table 2.

Serial Number	Localization of the Lesion	The duration of the disease at the examination of the sample:	Does the appearance of the disease resemble that of the classical actinomycosis?	Does the course of the illness up to this time resemble that of the classical actinomycosis with chronic course or is it an acute infection?	Remarks
1	The neck	2½ months			
2	The neck	1 month			
3	Regio mandibularis	1 month			
4	The neck	1½ months			
5	The neck	III for 4 months			
6	The neck	2 months			
7	Regio submaxillaris	½ month			
8	The cheek	1 month			
9	Multiple abscesses		Yes	Similar to typical actinomycosis	Strains 4 and 5 have been cultivated from 2 samples from the same pt.
10	The neck	4 months	Yes	Similar to the typical chronic actinomycosis	Pt. died later on. Diagnosis of autopsy: actinomycotic pyemia.
11	Regio submentalalis	¾ month	Some likeness	Probably acute	
12	The neck	1½ months	No	Resembles more an acute inflammation	Pt. treated with penicillin recovered by degrees.
13	Right Lung	About 6 months	Yes	Resembles typical actinomycosis of the lung	Strains 13. and 14 have been cultivated from 2 different samples from the same pt.
14			—		

15	Regio submaxillaris	About 2 months	Yes	Subacute	
16	The cheek	About 1 month	Yes	Acute	
17	The neck	1 year	Yes	Resembles the classical actinomycosis	
18	Border of the right mandible	2 months	Yes	Subacute lesions	
19	Regio submaxillaris	1 month		Indolent subacute formation of abscesses	
20	The cheek	1 month	Yes	Subacute	
21	Pars petrosa of os temporale	5 years	Yes	Resembles typical actinomycosis of the inner ear	Case published by <i>Jerlang</i> (1932)
22	The face	2 months			
23	Angulus mandibulae dextr.	3-4 months	Yes		
24	Angulus mandibulae dextr.	1 year	Yes	Chronic	
25					
26	Regio parotideo-masserica	About 4 months	Yes	Resembles actinomycosis	
27	Regio parotideo-masserica	1 month	Yes	Resembles actinomycosis	
28	The right cheek	3 weeks	Yes	Resembles the classical actinomycosis	
29	The right pleural cavity	2 months	No	Resembles an empyema pleura	Penicillin administered locally in pleural cavity. Recovered.

Clinical notes of the lesions from which the strains of Actinomyces have been cultivated.

fungi, which are identical with the microbe of the classical human actinomycosis, initially cultivated and described by Wolff & Israel in 1891, later on observed by many investigators, among others: Grillo (1898), Silberschmidt (1901), James Homer Wright (1905), Shiota (1909), Harbitz & Grøndahl (1910 & 1911), Colebrook (1920), Negroni & Bonfiglioli (1937), Lentze (1938), Dagny Erikson (1940) and Rosebury *et al.* (1944). In English speaking countries microbes belonging to that species are most frequently called *Actinomyces bovis*, sometimes also *Actinomyces israeli*, by which last name it will be referred to in the following, as I agree with those authors who consider this name to be the correct one.

The strains of that group yield characteristic colonies on blood agar under anaërobic conditions. They are raised, have a rough or nodular surface, and show a rosette form or an irregular outline. They are white. After 5 days of cultivation they usually attain a diameter of less than 1 millimetre. By cultivation in deep glucose agar shake culture, a characteristic band like growth occurs 5—10 millimetres below the surface of the medium; in glucose broth there is growth of a cauliflowerlike culture at the bottom of the tube, otherwise the medium remains entirely clear. On biochemical investigation the *Actinomyces* of that group are found to ferment the following carbohydrates, with the formation of acid and no gas: arabinose, xylose, rhamnose, mannose, lactose, raffinose, starch, amygdalin and inositol; as a rule sorbite is also, but slowly fermented, glycerine generally not in the course of 30 days (technique: Holm, 1930).

Group 2 includes some strains (21—27), all of which require anaërobic conditions as well as the presence of carbon dioxide to enable them to thrive on the surface of solid media. They have a great many characteristics in common with *Actinomyces israeli*, among other things, they develop colonies on blood-agar, completely resembling the above-mentioned; however, they differ from the microbes of group 1 in various ways: they give a viscous growth in broth and semifluid agar; they do not ferment arabinose, and are, as mentioned before, carbondioxidophile.

Apparently, Garrod has cultivated a similar strain from a patient with actinomycosis of the lung treated by Roberts, Tubbs & Bates (1945), who in their work report that the strain "was ... found to be atypical ... requiring CO₂ as well as anaërobiosis for growth: the colonies were viscous and web-like ...". Otherwise such *Actinomyces* are not referred to, which in itself is strange, considering that they often occur in pus samples sent to the Serum Institute for examination for *Actinomyces* (about 10 % of the strains isolated by the routine diagnostic work belong to that group). In case these *Actinomyces* should have been cultivated previously, they have probably been classified together with *Actinomyces israeli*, notwithstanding that without any specially thorough examination they differ so widely from that microbe as to justify regarding them as a distinct species. It may be

mentioned that from the clinical notes entered in table 2 it appears, that this type of *Actinomyces* will generally cause diseases similar to the classical actinomycosis.

2 strains (28 and 29) in group 3 differ widely from the Actinomyceetes of the 2 first groups; they grow on blood-agarplates, cultivated anaërobically, with complete circular, slightly convex colonies with a smooth, glistening surface; the colonies are clear or faintly milky, and consequently, show with a light reddish or grayish colour; they do not adhere to the medium. When examining the strains of that group by direct agar-microscopy (f. inst. by cultivation on ascitic agar) it is found that small typical mycelia are formed at the beginning of the growth; at an early stage, this manner of growth changes into angular growth (*Ørskov*, 1923), and greater colonies entirely resemble those of bacilli. Broth is clouded by strains 28—29, and among the carbon-hydrates mentioned before only arabinose and xylose and sometimes starch are fermented, but they yield poor growth in fermentation media produced with the peptone it has been possible to obtain during and after the war, and accordingly, it is difficult to examine them biochemically.

Actinomyceetes of that species are but little known. It must, however, be presumed that *Colebrook* (this author's group B) has seen such microbes in 1920. Previously, I myself have observed 3 similar strains (strain 1, 2 and 5 *Holm*, 1930), and *Lentze* (>S-form<- 1938) has no doubt also seen ray fungi of that group. It is possible that in 1944 *Rosebury* too has cultivated a microorganism of that kind.

In the 4th group 2 strains (30 and 31) are mentioned which are signified as >associates<. Later on I shall revert to the meaning of this. The 2 Actinomyceetes included in this group are not at all alike, nor do they resemble those described in groups 1, 2 and 3.

Experimental Methods.

Method: The penicillin sensitivity determinations have been performed in fluid medium in test-tubes.

Medium: from earlier investigations I knew that human pathogenic Actinomyceetes thrived poorly in nutrient broth, even under anaërobiosis, if singly placed germs were sown. If on the other hand, the broth contained some extract of blood, produced after a principle, almost simultaneously proposed by *Fleming* (1919) and *Martha Wollstein* (1919), there was good growth. An extract was prepared by mixing equal parts of horse blood and broth and then heating for some minutes at 100° C; in this way, the mixture coagulated, and a red liquid was produced, which was then centrifuged. The resulting clear fluid was then mixed with infusion broth (sterilized by filtration through Seitz filter) in the proportion of 1 part of extract to 9 parts of broth. This gave a medium with a final pH of 7.0—7.2.

The penicillin broth dilutions: on the days of experiment a series of solutions have been produced in a row of bottles by means of standardized dried penicillin*) in the above-mentioned broth. The penicillin concentrations

*) kindly supplied by Professor K. A. *Jensen*.

of these solutions have made up a geometric progression, the quotient being $\sqrt[4]{2}$. The highest penicillin concentration used has been one Oxford unit per millilitre, thereupon the concentrations $\frac{1}{\sqrt[4]{2}}, \frac{1}{2}, \frac{1}{2\sqrt[4]{2}}, \frac{1}{4}, \frac{1}{4\sqrt[4]{2}}, \dots, \frac{1}{64}$ have been employed. Of the penicillin broth dilutions produced, 2 millilitres were measured per test-tube. In each series of test-tubes with the various penicillin concentrations a control-tube was included, containing 2 millilitres of blood-extract-bouillon.

Either whole colonies or thin suspensions of *Actinomyces* have been used for inoculation of the tubes.

The whole colonies were taken from blood-agarplates which, after the inoculation had been incubated under anaërobic conditions, i. e. in hydrogen and carbon dioxide. Where nothing else is indicated, they are 5 days old, but in some of the experiments colonies have also been used, which were 4 or 3 days old only. The colonies were cut out of the surface of the agar by means of a flattened platinum wire, and then inserted into the tubes with the penicillin dilutions. One colony was sown in each tube.

The suspensions have been prepared from 5 day old colonies, taken from the same blood-agarplates as were the whole colonies. For each suspension 2—3 colonies were employed; they were placed on a sterile slide with a few drops of broth, and then crushed as carefully as possible by another sterile slide. The trifling quantity of suspension produced in this way was through a Pasteur pipette transferred into a broth-tube, where it was diluted with 2 millilitres of broth. With this thin suspension (of a number of singly placed germs and a few quite small lumps of culture, for the sake of convenience referred to as singly placed germs in the following) the test-tubes were inoculated, one drop in each tube. The same quantity of suspension was also sown on a blood-agar-plate, which was incubated anaërobically for 5 days. This plate showed how many living germs of *Actinomyces* had been sown in each tube. Generally, the number has been several hundred, at intervals, however, only a few hundred.

Incubation: all the tubes inoculated with *Actinomyces* were placed under anaërobic conditions, usually in jars filled with hydrogen; some strains, including f. inst. the strains of group 2, table 1, grew poorly or not at all, if carbon dioxide did not exist in the culture environment, and consequently, they were cultivated in jars, containing hydrogen with 2% of carbon dioxide. Simultaneously, on a small series of strains penicillin sensitivity tests were carried out by cultivation in hydrogen alone, and by cultivation in hydrogen with carbon-dioxide. The results of the 2 estimations turned out to be different, the penicillin regularly showed less inhibiting influence on the tubes cultivated in jars with carbon dioxide. At all events, I am of the opinion that the penicillin is most rapidly destroyed, when this gas is present, because the media turn acid. The penicillin sensitivity determinations performed in hydrogen alone therefore offer the most correct results. The hydrogen employed for the experiments is delivered from a cylinder containing the compressed gas (commercial), it contains about $\frac{1}{2}$ % of oxygen. The test-tubes are placed in the incubator at about 37° C.

The penicillin sensitivity of common bacteria is ordinarily determined by cultivation for 24 hours. However, this is not possible in connection with pathogenic *Actinomyces*, as these microbes do not show any visible growth in such a short time. It was therefore necessary to cultivate during a longer period, but was at first undecided as to the choice of time for reading off results. It was arranged to perform and take note of the results of the cultivation on the 10th, 20th, and 30th day; by this several readings were obtained, and the one might be used which was regarded being the best.

Table 3.

	Strains of Actinomyces	The tubes inoculated with	The colonies:		penicillin concentration in the broth-tubes (at the beginning of the experiment) stated in Oxford units per millilitre										
			Age in days	Diameter in mm.	1	2	1	1	1	1	1	1	1	1	0 (Control)
20/2-46	a	Suspension Whole colonies — —	3	0.09—0.15											+
			4	0.18—0.24											+
			5	0.30—0.36											+
	b	Suspension Whole colonies — —	3	0.09—0.15											+
			4	0.18—0.24											+
			5	0.21—0.30											+
	c	Suspension Whole colonies — —	3	0.09—0.15											+
			4	0.15—0.18											+
			5	0.18—0.24											+
	d	Suspension Whole colonies — —	3	0.09—0.12											+
			4	0.15—0.21											+
			5	0.21—0.30											+
	e	Suspension Whole colonies — —	3	0.09—0.15											+
			4	0.15—0.21											+
			5	0.21—0.30											+

Growth of 5 strains of Actinomyces israeli in penicillin broth-dilutions. Each strain has been examined in series of tubes, inoculated with suspension and with colonies, 3, 4 and 5 days old respectively. Anaerobic cultivation for 30 days. + indicates growth.

At a very early stage, it appeared from the investigations that penicillin of the weakest concentrations possessed a slightly inhibiting influence on the growth of the *Actinomyces*; in somewhat stronger concentrations it had a more powerful inhibiting effect, and in the strongest it was usually capable of preventing the growth entirely. It seemed to me that the best way of expressing the penicillin sensitivity was by stating in which tubes, the penicillin was quite capable of preventing growth, and in which growth occurred in spite of the presence of penicillin. Consequently, I have in the tables, entered a + for the tubes exhibiting growth at all; if, corresponding to a tube, no sign has been made, it means that no growth has been shown on the 10th, 20th, and 30th day in the tube concerned. Besides, I can point out that I have never observed growth in any tube on the 30th day, where this did not also exist clearly on the 20th day.

Certainly, the method described above for penicillin sensitivity determinations deviates considerably from the usual ones. In comparison, it was accordingly desirable to carry out a penicillin sensitivity test in quite a similar manner, and in the same media, on some well-known microbe. I decided to examine 20 strains of *Staphylococci* (Oxford H *Staphylococcus*, and 19 other *Staphylococci*, all cultivated from patients with furunculosis).

The tubes in these series have been inoculated with suspensions only. Some thousand germs have been sown per tube, they have been cultivated aërobically. The staphylococcal test-tubes have been read after 1, 2, 5, 10, and 15 days. The reading was always equivalent for the same strain on the 5th, 10th, and 15th day.

Preliminary Experiments.

Before the actual penicillin sensitivity tests were performed, some experiments were made on the technique using some strains of *Actinomyces* chosen at random. Only one of these experiments will be mentioned. It comprised 5 strains of *Actinomyces israeli*, each of which was sown in 4 rows of penicillin-broth-dilutions with a control-tube for each. The first series of tubes was inoculated with suspension, the last 3 series with whole colonies 3, 4 and 5 days old, respectively. The cultivation took place in hydrogen-atmosphere, and with readings after 10, 20, and 30 days. The results have been stated in table 3. It is obvious from the experiment, that unequal results are obtained, the tubes being inoculated differently. If the inoculations are carried out with colonies being about 1/3 millimetre of diameter (i. e. 5-day old colonies), the resistance of the strains are found to be from 4 to about 22 times as great as when being inoculated with a thin suspension.

Table 4.

	Staphylococcal strains	Penicillin concentration in the broth-tubes (at the beginning of the experiment) stated in Oxford units per millilitre								
		$\frac{1}{4}$	$\frac{1}{4 \frac{1}{2}}$	$\frac{1}{8}$	$\frac{1}{8 \frac{1}{2}}$	$\frac{1}{16}$	$\frac{1}{16 \frac{1}{2}}$	$\frac{1}{32}$	$\frac{1}{32 \frac{1}{2}}$	$\frac{1}{64}$ 0 (Control)
20/2-46	Oxford-H							+	+	+
	2					+	+	+	+	+
	3									+
	4						+	+	+	+
	5						+	+	+	+
16/3-46	Oxford-H							+	+	+
	2					+	+	+	+	+
	3							+	+	+
	4						+	+	+	+
	5							+	+	+
2/4-46	Oxford-H							+	+	+
	2						+	+	+	+
	3								+	+
	4						+	+	+	+
	5							+	+	+
10/4-46	Oxford-H							+	+	+
	2					+	+	+	+	+
	3							+	+	+
	4							+	+	+
	5						+	+	+	+
22/6-46	Oxford-H							+	+	+
	2						+	+	+	+
	3							+	+	+
	4							+	+	+
	5						+	+	+	+
16/7-46	Oxford-H							+	+	+
	2					+	+	+	+	+
	3							+	+	+
	4						+	+	+	+
	5						+	+	+	+
1/5-47	Oxford-H							+	+	+
	2					+	+	+	+	+
	3							+	+	+
	4						+	+	+	+
	5					+	+	+	+	+

Penicillin resistance experiment on 5 staphylococcal strains repeated on 7 different days. Aërobic cultivation for 15 days. + signifies growth.

Experimental Results.

Having seen the results of the experiment just mentioned I decided to examine my collection of indisputably human-pathogenic strains of Actinomyces. It was not possible, however, to examine all of them at once, consequently, I was compelled to divide the investigation into several experiments. Some strains of Actinomyces and some of Staphylococcus were now examined on each day of experiment. Each strain of Actinomyces was examined in 2 series of tubes with penicillin-dilutions. One series was inoculated with whole colonies and the other with suspension. In table 6 is recorded how the incubation of the inoculated test-tubes has been carried out, seeing that in a special column it is indicated, whether this has taken place in hydrogen or in hydrogen with carbon dioxide (2 %). The strains of Staphylococcus were inoculated only in one series of tubes per strain, and incubated aëroically as mentioned before.

To find out whether the conditions of experiment have been uniform on the different days, I inoculated, each time an experiment was made, 5 specific strains of Staphylococcus (Oxford-H, 2, 3, 4, and 5) in series of tubes with penicillin-dilutions. Table 4 shows the results of the sensitivity determinations of those 5 Staphylococci on the different days of experiment. The results are as much alike as can be expected.

I am of the opinion that from table 4 it may be concluded that it is permitted to compare the penicillin sensitivity determinations of the different days, and I have, accordingly, placed the results of the investigations on the 20 Staphylococci in one table (no. 5), and those of the Actinomyces in another table (no. 6). In the latter, 2 estimations are given for each strain of Actinomyces, the results of inoculations with suspension, and those of whole colonies, respectively.

From table 6 (left half) it will be seen that all strains of Actinomyces examined are sensitive to penicillin, if suspensions i. e. singly placed germs (or very small lumps of culture only) were used for the resistance-test for the inoculation of the tubes. None of the 31 strains yielded growth in broth containing $\frac{1}{8\frac{1}{2}}$ i. e. about $\frac{1}{12}$ Oxford units of penicillin per millilitre, and only 6 of them grew in a medium where at the beginning of the experiment there was $\frac{1}{16}$ Oxford units per millilitre.

By comparing the results of this half of table 6 with those recorded in table 5 concerning the Staphylococci, it is found that on the whole, Actinomyces and Staphylococci are of the same sensitivity to penicillin, thin suspensions being used for the resistance-estimations.

If the left and right half of table 6 are compared it is obvious that whole colonies of Actinomyces appear to be more resistant than singly placed germs. Some of the whole colonies examined f. inst. those of

strain 14 thus thrived in broth which contained one unit of penicillin per millilitre at the beginning of the experiment, whereas singly placed germs of the same strain were killed in all concentrations below $\frac{1}{32}$ units. A few strains exhibit a vastly greater difference in resistance between whole colonies and singly placed germs, most of the ray fungi examined, however, show less difference.

Looking at the right half of table 6 alone, it will at once be noticed that the much resistant whole colonies are exclusively to be found amongst the strains of group 1 (1—20; the strains of *Actinomyces israeli*) and in group 4 (30—31), whereas the whole colonies of *Actinomyces* comprised in group 2 (21—27) and group 3 (28—29) must be characterized as being fairly sensitive to penicillin.

Table 5.

	Staphylococcal strains	Penicillin concentration in the broth-tubes (at the beginning of the experiment) stated in Oxford units per millilitre									
		$\frac{1}{4}$	$\frac{1}{4\sqrt{2}}$	$\frac{1}{8}$	$\frac{1}{8\sqrt{2}}$	$\frac{1}{16}$	$\frac{1}{16\sqrt{2}}$	$\frac{1}{32}$	$\frac{1}{32\sqrt{2}}$	$\frac{1}{64}$	0 (Control)
20/2-46	Oxford-H							+	+	+	+
	2					+	+	+	+	+	+
	3									+	+
	4						+	+	+	+	+
	5						+	+	+	+	+
16/3-46	6					+	+	+	+	+	+
	7							+	+	+	+
	8						+	+	+	+	+
	9					+	+	+	+	+	+
	10						+	+	+	+	+
2/4-46	11							+	+	+	+
	12						+	+	+	+	+
	13							+	+	+	+
	14							+	+	+	+
	15							+	+	+	+
10/4-46	16						+	+	+	+	+
	17						+	+	+	+	+
	18						+	+	+	+	+
	19					+	+	+	+	+	+
	20						+	+	+	+	+

Penicillin resistance experiment on 20 different strains of *Staphylococcus aureus*. Aërobic cultivation for 15 days. + signifies growth.

It has been stated several times in the before mentioned — with reference to the experiments recorded in the tables 3 and 6 — that whole colonies of *Actinomyces* appear to be more resistant than singly

Table 6 (left half).

	Strains of Actinomyces	Cultivated in	Inoculated with suspension Penicillin concentrations in the broth-tubes (at the beginning of the experiment) stated in Oxford units per millilitre.									
			1	1	1	1	1	1	1	1	0	
			4	4 1/2	8	8 1/2	16	16 1/2	32	32 1/2	64	(Control)
10/4-46	1	H ₂					+	+	+	+	+	+
—	2	—						+	+	+	+	+
—	3	—							+	+	+	+
—	4	—						+	+	+	+	+
22/6-46	5	H ₂ +CO ₂						+	+	+	+	+
10/4-46	6	H ₂							+	+	+	+
—	7	—						+	+	+	+	+
26/3-46	8	H ₂ +CO ₂						+	+	+	+	+
20/2-46	9	H ₂					+	+	+	+	+	+
16/3-46	10	—					+	+	+	+	+	+
—	11	—						+	+	+	+	+
—	12	—							+	+	+	+
—	13	—							+	+	+	+
—	14	—								+	+	+
2/4-46	15	—							+	+	+	+
—	16	—								+	+	+
—	17	—									+	+
—	18	—									+	+
16/7-46	19	—								+	+	+
—	20	—									+	+
16/7-46	21	H ₂ +CO ₂						+	+	+	+	+
—	22	—						+	+	+	+	+
—	23	—						+	+	+	+	+
—	24	—						+	+	+	+	+
—	25	—						+	+	+	+	+
—	26	—						+	+	+	+	+
22/6-46	27	—					+	+	+	+	+	+
22/6-46	28	H ₂ +CO ₂					+	+	+	+	+	+
—	29	—										+
22/6-46	30	H ₂ +CO ₂							+	+	+	+
—	31	—									+	+

Growth of 31 strains of Actinomyces in penicillin broth-dilutions, cultivated ated with suspension and with whole colonies, respectively.

placed germs. This cautious phrasing has been chosen on purpose. Actually, the experiments referred to in these tables allow no definite conclusion as to the relation between the penicillin-resistance of whole colonies and that of germs, as the tubes of the 2 series have not been inoculated with the same quantity of culture; the tubes inoculated with whole colonies contained 40—50 times as much culture of *Actinomyces* as those having been sown with suspension.

As the question, however, of the resistance of whole colonies is of great interest, one more experiment was made with some strains of *Actinomyces israeli*. Each strain was sown in 3 rows of penicillin broth-dilutions. The first and third rows were inoculated just as previously with thin suspensions and with whole colonies (of about 1/3 millimetre of diameter), respectively. The second row was inoculated with a suspension containing the germs of a whole colony of *Actinomyces* per drop (= the inoculum used for each tube). The tubes were incubated and read as in earlier experiments. The results are recorded in table 7.

Comparing here the results of the penicillin sensitivity determinations in the 2nd and 3rd series of tubes for each strain, it is seen that whole colonies of *Actinomyces* are actually more resistant than singly placed germs, and as regards some strains (10, 14, and 17) much more resistant.

Furthermore, it is obvious from the experiment that penicillin sensitivity test performed on singly placed germs yields nearly the same results, whether the individual tubes are inoculated with little or much culture.

On comparing the results of the tables 6 and 7 it will moreover be seen that, strictly speaking, penicillin sensitivity tests carried out on the same strain on 2 different days of experiment give the same results. This fact seems to hold good both when suspension was used for the investigation, and when the tubes were inoculated with whole colonies.

Some Comments on the Penicillin Treatment in Actinomycosis.

From the experiments mentioned before it appeared that all 31 anaërobic indisputably human-pathogenic strains of *Actinomyces* examined are sensitive to penicillin, and to the same degree as are *Staphylococci*. Consequently, there is good reason to expect penicillin to have a favourable — perhaps curative — effect on actinomycosis, and it is my opinion that every case of this disease, threatening the patient's life, ought to be treated with this substance, if the cause of the actinomycosis is some anaërobic *Actinomyces*.

Penicillin can, as in the case of any other surgical suffering, be employed locally or generally, and probably in actinomycosis it ought to be administered in both ways (in many cases combined with an adequate surgical treatment). I shall not go further into the method

Table 7.

	Strains of Actinomyces	The tubes inoculated with	Diameter of the colonies in millimetre	Penicillin concentration in the broth-tubes (at the beginning of the experiment) stated in Oxford units per millilitre.												
				1	1	1	1	1	1	1	1	1	1	1	1	0 (Control)
1/5-47	9	1 Thin suspension 2 Dense — 3 Whole colonies	0.21—0.27	+	+	+	+	+	+	+	+	+	+	+	+	+
	10	1 Thin suspension 2 Dense — 3 Whole colonies	0.27—0.33	+	+	+	+	+	+	+	+	+	+	+	+	+
	13	1 Thin suspension 2 Dense — 3 Whole colonies	0.24—0.30	+	+	+	+	+	+	+	+	+	+	+	+	+
	14	1 Thin suspension 2 Dense — 3 Whole colonies	0.27—0.33	+	+	+	+	+	+	+	+	+	+	+	+	+
	16	1 Thin suspension 2 Dense — 3 Whole colonies	0.21—0.27	+	+	+	+	+	+	+	+	+	+	+	+	+
	17	1 Thin suspension 2 Dense — 3 Whole colonies	0.27—0.33	+	+	+	+	+	+	+	+	+	+	+	+	+

Growth of 6 strains of *Actinomyces israeli* in penicillin broth-dilutions. Each strain has been examined in 3 series of tubes inoculated with thin and dense suspension and with whole colonies, respectively. The penicillin sensitivity tests in the 2 last series of tubes of each strain are directly comparable, as the tubes in these series have been inoculated with the same quantity of culture per tube. Anaerobic cultivation for 30 days. + signifies growth.

by which the local treatment is to be performed, this fact is in each individual case dependent on the anatomic relations of the disease; but being of the opinion that some of the experiments made *in vitro* give cause for it, I want to advance some remarks on the dosage of the general treatment. The initial point for these considerations is the experience of the clinicians that, doses having been sufficient for treatment of patients with staphylococcal bacteriemia, often failed in patients with actinomycosis. In itself this is strange, when it is remembered that the 2 microbes were equally sensitive to penicillin; but, on the other hand, it has been noticed so frequently that there can be no doubt of it. It is not easy to give any reason for this failure, probably, there are several reasons, and they are most likely different from one patient to another; however, it will be mentioned, as is well known, that it is difficult for chemotherapeutics and antibiotics to penetrate into the abscesses themselves in surgical infections, especially large abscesses (see *Meleney*, 1944, a). At all events, I should think that it is also important, that the ray fungi found in the pus of the patients occur in the shape of small colonies (the typical »sulphur granules«). We *do know* from the experiments referred to in the tables 5, 6 and 7 that by experiments *in vitro* the whole colonies of some strains of *Actinomyces* are essentially more resistant than singly placed germs (and than *Staphylococci*), it should accordingly not be quite unjustified to *believe* that something similar holds good for the granules contained in the pus of the suppurative lesions of the patients.

Consequently, I am of the opinion that it must be justifiable to put forward the working hypothesis, that actually the resistance of the *Actinomyces* in the body are to be given by the resistance of the granules. The experiments made *in vitro* report nothing of the strength of the resistance *in vivo*, possibly not even an exact figure for the true *in vitro* resistance of the whole colonies, seeing that they have been performed in medium in which the penicillin concentration declines rather quickly on account of the natural disintegration of the penicillin, but I should think that it more or less approaches the resistance found for whole colonies without being quite as great, however.

Supposing it is true that the resistance *in vivo* of the ray fungi is given by the resistance of the granules, and that the latter approaches the resistance for whole colonies found in this work, then it must be expected — (refer to the right half of table 6) — that cases of illness developed by different types of *Actinomyces* are to be treated with different doses of penicillin.

In this case there will be good reason to suppose that patients having been infected by microbes of the types classified in this work in the groups 2 and 3, are influenced favourably, and will most likely recover, by the use of not very large doses of penicillin.

On the other hand, it cannot be expected to cure patients having

been infected by *Actinomyces israeli*, i.e. cases of classical actinomycosis, unless very large doses are administered, as whole colonies of those microbes exhibit an even fairly distinct resistance as far as the main part of them are concerned.

The hypothesis framed can be proved only by clinical observations. Already, a few such observations have been published which seem to support the hypothesis and seeing that the question is of such great interest they will be referred to.

First there are reports that 2 patients with actinomycosis of the lung, caused by group 2 *Actinomyces*, recovered by the use of not especially large doses of penicillin. Patient 1 has been treated by *Roberts, Tubbs & Bates* (1945). This was a 22-year-old man who was at first treated for 15 days with 120,000 units per diem, and reacted dramatically to this dosage, as he was transformed from a dying man to one who was apparently in good health. However, 3 weeks later he had a relapse. Then he was treated for 28 days with 200,000 units per diem, and recovered. The *Actinomyces* from that patient has been cultivated and examined by *Garrod*.

Patient 2, infected by a group 2 strain, has been treated by *Lachmann* (1946). This was a 9-year-old girl who was treated with 160,000 units intramuscularly, and locally (in a thoracic abscess) 10,000 units daily for a fortnight. The intramuscular treatment was continued with reduced dosage for 4 weeks, 4 million units were administered in all. The patient made such a rapid and complete recovery that *Lachmann* was of the opinion that considerably smaller quantities of penicillin might have attained the object. (A dosage of 160,000 units for a 9-year-old girl corresponds nearly to one of 400,000 units for a grown-up, if the weight is considered in the calculation). I myself have examined the strain of *Actinomyces* from *Lachmann's* patient.

Secondly, reports have been published on cases of actinomycosis which have not recovered in spite of a fairly intensive penicillin treatment. Such cases are for example, mentioned in *Fleming's* book: »Penicilline«, in which *Vaughan Hudson* (1946) reports some cases which have not recovered despite the use of 500,000 units of penicillin per diem for 21 days; even if there is no pure-cultivation and specie determination of the *Actinomyces* from *Hudson's* patients his observations appear to confirm the existence of cases of actinomycosis which cannot be cured by even a rather strong penicillin-treatment, and I would suppose that one is also entitled to count on *Hudson's* observations as being in favour of the hypothesis that the resistance of the *Actinomyces* in the body will be indicated by the resistance of the granules. Besides, experiences of exactly this kind were the cause of the hypothesis being put forward.

From the few experiences existing it is not easy to indicate which penicillin doses ought to be given to the patients with actinomycosis

caused by the different types, but I should think that cases of disease produced by Actinomyces of group 2 may in many cases be cured by the administration of 2—300.000 Oxford units of penicillin per diem for a comparatively short time (3—4 weeks); most likely, a similar dosage will frequently prove effective, when it applies to patients having been infected by ray fungi of group 3. On the other hand, patients being infected by Actinomyces israeli should to all appearance usually be treated by considerably larger doses. There is hardly any reason to expect any effect on those patients, unless 6—800.000 units are given per diem during a rather long period (5—6 weeks), and after all it is possible that in a number of cases considerably larger doses are needed for an even longer time to attain the objective: the patient's recovery.

If a bacteriological examination of the pus from patients are performed together with pure-cultivation and specie determination of the strains of Actinomyces before the penicillin treatment is instituted, it is possible to proceed according to the above, when fixing the doses. No classification being employed before the treatment is started, penicillin doses must be given that will suffice to influence Actinomyces israeli, which, in Denmark at least, appears to be the most frequent cause of actinomycotic infections.

Can all patients with actinomycosis then be expected to recover, also those suffering from actinomycosis of the lung, abdominal actinomycosis, and from actinomycotic pyemia, if only sufficiently large doses of penicillin are given and during a sufficiently long period? It is difficult to answer this question; but I am obliged to think that in quite a number of cases it must be possible to obtain a favourable effect of the penicillin treatment, even on those, nearly always, fatal illnesses.

However, actinomycosis is a strange disease, and many surprises may occur. If pus from a great many actinomycosis-patients is examined it is found that in addition to the Actinomyces other microbes are always present. Those bacteria by German investigators called »Begleitbakterien«, will, in the following, be referred to as »associates«. *Klinger* (1912) was the first one who has drawn attention to the frequent presence of such bacteria. Later on they have been found by several investigators, among others by *Colebrook* (1920), *Bayne-Jones* (1925), *Negroni* (1934), *Negroni & Bonfiglioli* (1937), *Goldsworthy* (1938), and *Clemens* (1940). *Bates* (1935) maintains that »associates« are always to be found on examination of pus from patients with actinomycosis. He has examined 29 samples, and is of the opinion that the »associates« are of importance for the outset of the disease. Likewise, *Lentze* (1939) has continuously been able to point out a mixed infection in actinomycosis. He has examined samples from 65 patients and states that there are always one or several kinds of

anaërobic bacteria; I myself can report that I do not remember among samples from 6—700 actinomycosis-patients ever having seen a single one where anaërobic Actinomyces were found alone.

What influence may these »associates« have on the results of penicillin treatment of patients with actinomycosis?

In the most favourable cases the »associates« are sensitive to penicillin, and are then killed by this substance together with the Actinomyces; if this happens the patient will recover, and in this case it must be said that the presence of the associating bacteria has had no influence on the results of the treatment.

In most cases, however, the »associates« are Gram-negative small anaërobic bacilli or coccobacilli. In advance, it will be expected that these microbes are resistant to penicillin, since this antibiotic acts especially on Gram-positive microbes. If an actinomycosis patient is treated where the Actinomyces are found together with such penicillin resistant »associates«, the most favourable result to be expected is to succeed in killing off all Actinomyces existing in the diseased organism, whereas the »associates« remain. In this way, however, a great deal has been gained for the patient, as, if concerned with an actinomycosis of the lung, the disease has been transformed from a fatal lesion to one which may be accessible to ordinary therapy (medical or surgical).

In most unfavourable cases the »associates« can produce penicillinase; it goes without saying that in those cases no greater effect of the penicillin on the Actinomyces may be expected, unless the »associates« can be destroyed by other therapy (chemotherapy, other antibiotics).

It is seen in the section on the strain examination, that I have only investigated into the penicillin sensitivity of anaërobic Actinomyces. The considerations just mentioned on the treatment of actinomycosis are therefore valid only for diseases produced by those anaërobic microbes.

Certain species of aerobic acid-fast Actinomyces (Actinomyces or Nocardia asteroides and gypsoides) however, are also capable of producing disease in man (nearly always more or less chronic pneumonias). I have had no opportunity of examining the penicillin sensitivity of microbes belonging to those species, and consequently, I cannot have any well-founded opinion, as to whether diseases of this kind ought to be treated by penicillin. According to the literature available (see: Drake, 1946) there is, however, good reason to believe that the aerobic acid-fast Actinomyces (Nocardia asteroides and gypsoides) are penicillin resistant to a high degree. Regarding this fact, these illnesses cannot be expected to be influenced favourably by penicillin. It is possible on the other hand, that in some of these cases sulphonamide treatment may be advantageous (Benbow et al., 1944).

Summary.

29 (31) indisputably human-pathogenic anaërobic Actinomyces have been isolated from 27 patients, classified in species, and examined for penicillin sensitivity.

If suspensions of the ray fungi were used for the sensitivity tests, the Actinomyces were found to have the same degree of sensitivity as Staphylococci. If whole colonies of Actinomyces were used, the strains were seen to fall into 2 groups. In one group the colonies were much more resistant than Staphylococci, in the other, they were but little more resistant than these microbes.

The hypothesis is put forward that the resistance of the Actinomyces in the body will be indicated by the resistance of the typical »sulphur granules« existing in the pus, and it is supposed that this resistance more or less approaches that found for whole colonies in the laboratory.

By means of this hypothesis, it is explained that on the administration of a comparatively short treatment with rather small doses of penicillin some patients with actinomycosis have an easy recovery, whereas in other cases the use of very large doses during a longer period must undoubtedly be demanded.

Patients with actinomycosis in the organs ought to be treated with penicillin if the cause of the disease is an anaërobic Actinomyces.

A temporary dosage diagram is made, in which is indicated which penicillin doses should be given to patients with actinomycosis produced by the different species.

It is mentioned that anaërobic Actinomyces in the disease products are always found together with other microbes, which may be called »associates«. The importance of those bacteria on the results of the penicillin treatment is discussed.

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Finally I wish to express my appreciation of the valuable technical assistance given by Miss Olga Benedicte Bruhn.

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EXPERIMENTAL INVESTIGATIONS INTO THE RELATION BETWEEN SYPHILIS AND CARCINOMA

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In 1933 *Castiglioni* reported investigations showing that skin carcinoma may be induced more rapidly in experimental animals infected with syphilis than in non-syphilitic animals. Following percutaneous application of tar to the inside of the ear of 17 rabbits previously infected with syphilis intravenously and 17 non-infected rabbits he found that the precancerous and malignant changes developed earlier in the syphilitic than in the non-syphilitic rabbits.

Truffi & Cerutti infected 30 white mice, aged 3—4 months, by implanting small pieces of chancre (type Truffi) below the skin. Three months later they began painting syphilitic mice and controls with tar every third day. Like *Castiglioni* they found that the malignant growths appeared earlier in the syphilitic than in the control mice.

In 1942 *Bessemanns & Maisin* repeated *Castiglioni's* experiment, using, however, mice instead of rabbits and carcinogenic hydrocarbons instead of tar. *Bessemanns'* material consisted of 30, 20, and 126 experimental and 50, 30, and 110 control mice. A fresh piece of testicular tissue derived from rabbits and containing an ample amount of motile spirochetes (type Gent) was implanted into the experimental mice subcutaneously in the region of the genitals. Four months later the mice in the first and second series were painted on the back with a solution of benzpyrene, $\frac{1}{2}$ per cent., 25 times in all, and the mice in the third series with a solution of methyleholanthrene, $\frac{1}{4}$ per cent., 20 times in all. Each painting was made with 2 drops of the solutions, containing 6.25 and 2.5 mg., respectively of the carcinogenic hydrocarbons. In neither case did they find any difference in the rate and frequency of the development of skin carcinoma in the syphilitic mice and the controls. *Bessemanns* expresses the desire that the problem be elucidated by further investigations, as mice are not particularly

well-suited for these experiments, owing to the fact that in mice *Treponema pallidum* reproduces as in a culture, and the mice exhibit no detrimental effect of the infection. In rabbits, on the other hand, these organisms cause pathological changes resembling the syphilitic manifestations in man.

Since no further investigations into the relation between syphilis and carcinoma appear to have been made, the writer has attempted to reproduce the experiments. For the reasons stated by Bessemanns rabbits were chosen as experimental animals.

The carcinogenic hydrocarbon chosen was 9,10-dimethyl-1,2-benzanthracene which according to *Berenblum* (Cancer Research 1945) must be considered the most effective one in the case of rabbits. *Berenblum* used a 1 per cent. solution in benzene. Twice weekly he painted 5 young albino rabbits on the inside of the right ear with 8 drops of the solution using a Pasteur pipette. The first tumours appeared after 5½, 6, 7, and 9 weeks of painting respectively. In one rabbit the excrescences remained benign until the experiment was concluded (26th week), whereas the others exhibited definite objective signs of malignancy after the lapse of 16, 16, 19, and 21 weeks. Histological examinations confirmed the malignancy.

Writer's Investigations.

- (1) Investigations into the carcinogenic effect of 9,10-dimethyl-1,2-benzanthracene on the strain of rabbits kept at the Institute in order to find the optimum concentration.
- (2) Investigations into the difference, if any, in the rate of development of tumours in syphilitic and non-syphilitic rabbits.

1. This experiment was carried out with young white rabbits of a strain bred at the Institute, a total of 4 litters of 4 rabbits each (9 ♂, 7 ♀). A rabbit of each litter was painted with 9,10-dimethyl-1,2-benzanthracene, 2 per cent., 1 per cent., ⅔ per cent., and ⅓ per cent. respectively in benzene, with the technique of *Berenblum*.

The rabbits were kept under identical conditions, receiving the same diet and living in cages not directly exposed to sunlight due to the inactivation of 9,10-dimethyl-1,2-benzanthracene by light.

The hyperæmic and oedematous changes described by *Berenblum* were observed after each painting, but did not persist during the intervals. Following 2—3 paintings there was beginning desquamation and epithelial thickening.

Table I sets out the number of days elapsing from the start of the experiment until the appearance of the first tumour, reckoning only tumours at least the size of a pea, as the quite small swellings are not constant. It will be seen that the concentration 1 per cent. induces tumours earlier than the 2 per cent. solution, i. e. in 31—48 days compared with 35—73 days in the case of the 2 per cent. solution.

Table 1.
Number of Days before the First Appearance of a Tumor
(at least of pea-size).

	2 %	1 %	$\frac{1}{3}$ %	$\frac{1}{10}$ %
litter A	64	38	64	112
» B	35	35	35	98
» C	73	31	112	112
» D	43	48	98	112

$\frac{1}{3}$ per cent. induced tumours in 3 cases following painting for 35 to 98 days, but in one case (litter C) no tumours developed.

Following painting with the weakest concentration ($\frac{1}{10}$ per cent.) no tumours were induced in 3 cases, but in one case (litter B) a swelling, as large as a pea, was observed after 98 days.

It will be seen from Table I that the different litters show a different induction time. Thus, litter B has an average latent period of 35 days in the case of the first 3 concentrations, whereas litters A and D have a latent period of 55 and 63 days respectively.

Table II presents all 4 litters 38, 64, 92, and 112 days after the experiment was started. There is a distinct difference between the growth of tumours obtained with the strong solutions (2 and 1 per cent.) compared with the weak solutions ($\frac{1}{3}$ and $\frac{1}{10}$ per cent.).

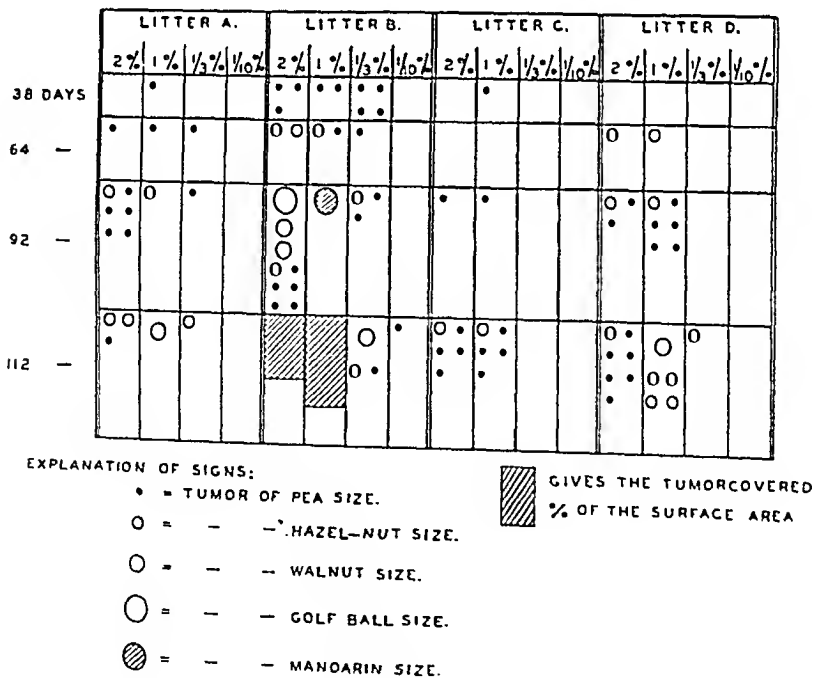


Table 2.
Graphical Survey of the Results Obtained in the Four Litters.

It will be seen from the graphic representation in the table that in the rabbits painted with the strong concentrations the tumours grew to such an extent that the neoplastic mass in one case covered two-thirds of the inside of the ear.

Comparing the size and extent of the swellings at the end of the experiment in the rabbits painted with the strong solutions, one finds that in 3 cases (litters A, B and D) painting with 1 per cent. resulted in the largest growths, whereas in 1 case (litter C) the growths were alike in the two rabbits painted with 1 per cent. and 2 per cent. respectively.

In other words, the 1 per cent. solution does not only induce the first tumours earlier than 2 per cent., but when extended over a long period the former appears to induce larger tumours than the latter. There was no difference between the induction time of tumours in males and females.

Histological examination revealed *spinocellular carcinoma* in 8 cases (painted with 1 and 2 per cent.). Biological findings support the diagnosis, as 6 cases exhibited invasive growth through the aural cartilage. In the remaining 4 cases technical mishaps prevented microscopical examination. Metastases were not found in any case.

2. The second part of the investigations was also carried out with young white rabbits of a strain bred at the Institute. The material comprises a total of 33 males and 30 females derived from 14 litters. The males were infected with *Treponema pallidum*, type Gent*) by means of intratesticular injection of a suspension of *Treponema pallidum*, $\frac{1}{2}$ cc., containing 10—20 motile organisms in each field of the microscope. In all cases the infection took in the course of 4—6 weeks. The testes swelled and became as hard as cartilage, and in 6 cases ulcerating chancres were observed. In 7 syphilitic rabbits, selected at random, biopsy specimens revealed numerous spirochetes, and pieces of testicular tissue removed and implanted into other rabbits transmitted the infection.

As soon as the syphilitic infection was manifest, in the course of 8—10 weeks, the painting of the experimental and control rabbits was started with 9,10-dimethyl-1,2-benzanthracene, 1 per cent. in benzene. The technique was the same as that used in the earlier experiments, the inside of the right ear being painted twice weekly, each time with 8 drops of the solution.

Table III sets out the time at which the experimental and control rabbits acquired tumours showing evidence of malignancy upon microscopical examination. After 7 weeks of painting 21 males and 15 females exhibited malignant tumours; after 9 weeks 24 males and 19 females. After 16 weeks of painting all the survivors (3 rabbits died

*) To Professor *Bessemanns*, Gent, my thanks are due for kindly supplying the *Treponema pallidum* of type Gent.

Table 3.

Litter No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Number of weeks after painting was started } 4															
5													+	+	1 3
6						+	+						+		1 1
7	+	+	+	+	+										5 2
8	+	+	+	+			+	+	+	+	+	+			14 9
9							+	+							3 3
10	+														1
11						+	+	+		+			+	+	4 6
12		+	+	+											1 2
13															
14					+										1
15															
16						+									1

+ Mier. exam. showing spinocellular carcinoma.

+. 2-6 weeks earlier the mier. exam., now showing spinocellular carcinoma, had been negative.

30 28

before exhibiting tumours) showed malignant tumours except females Nos. 48 and 49. These figures show a not significant predominance of the syphilitic rabbits with regard to the rate at which the cancerous tumours arise. Accordingly, the result of these investigations does not support the observations of *Castiglioni* and *Truffi*.

Summary.

Four litters of 4 rabbits each were painted with 9,10-dimethyl-1,2-benzanthracene, 2 per cent., 1 per cent., $\frac{1}{3}$ per cent., and $\frac{1}{10}$ per cent. in benzene, with the technique of *Berenblum*. The 1 per cent. concentration did not merely induce the earliest tumours, but also those which grew largest during the experimental period.

Thereupon the writer attempted to reproduce *Castiglioni's* experiments in which the latter found that carcinomatous growths developed earlier in syphilitic than in non-syphilitic animals. A total of 33 male rabbits infected with syphilis and 30 female controls were painted for 16 weeks with 9,10-dimethyl-1,2-benzanthracene, 1 per cent. in benzene. After the lapse of 7 weeks 21 males and 15 females exhibited malignant tumours and after 9 weeks of painting 24 males and 19 females. Accordingly, no significant difference could be found between the induction time of tumours in syphilitic and non-syphilitic rabbits.

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Fig. 1.

Rabbit no. 3. Tumor on the inside of the right ear after 7 weeks of painting with 9,10-dimethyl-1,2-benzanthracene, 1 per cent.



Fig. 2.

Biopsy specimen from the tumor in no. 3 (after 7 weeks of painting with 9,10-dimethyl-1,2-benzanthracene (1 per cent)) ($\times 25$).



Fig. 3.
Same as Fig. 2 ($\times 110$).



Fig. 4.
Rabbit no. 3. Invasive growth through the aural cartilage to the outside of the ear, (after 11 weeks of painting with 9,10-dimethyl-1,2-benzanthracene, 1 per cent).



Fig. 5.

Rabbit no. 8005: Tumor after 26 weeks of painting with 9,10-dimethyl-1,2-benzanthracene, 1 per cent.

I. PATHOGENIC-APATHOGENIC TRANSFORMATION OF SALMONELLA TYPHIMURIUM

By O. Maaloe.

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Part I.

As an introduction to this paper it is necessary to analyse the general conception of »pathogenicity«, and in so doing to define the different factors which together determine whether a microorganism is pathogenic or not. — In this analysis we deal with the acute phase of bacterial infections in normal *i. e.* non-immunized organisms. This means that allergic and other immunological phenomena will be left out of consideration.

The relation between a microorganism and a macroorganism can be characterized by the pathogenicity of the microorganism with respect to the macroorganism in question. This pathogenicity can be generally described as a combination of three factors, which logically may be treated as independent, though, as we shall see later, they are often more or less correlated.

The three factors are:

A. The *Infectivity* of the bacterium; *i. e.* the faculty determining to what degree the bacteria are able to penetrate an epithelial wall of the macroorganism. After specifying the site of invasion, we may express the infectivity by means of the percentage of bacteria which in a given time penetrate into the underlying tissue.

B. The bacterium's faculty for multiplication *inside* the invaded macroorganism, facing its antibacterial forces. For this factor we use the term *Invasiveness*. After specifying the tissue in which growth takes place, we can thus describe the invasiveness of a bacterium by the means of its growth-curve.

C. The third and last factor entering the conception of pathogenicity is the *Toxicity* of the bacterium. — By toxicity we should understand in the most general way, the »dangerousness« of the bacterium *i. e.* the degree to which the metabolic processes of the bacterium, or its

possible toxic products, interfere with the normal functions of the infected organism. — Quantitatively, toxicity may be expressed by the number of bacteria which must enter or be produced inside the organism in order to evoke certain pathological changes.

The three factors A, B and C have, so far, been treated as independent. This is of course a simplification, and, as mentioned above, they may well be correlated: It is, for instance, usually found that the toxic effect on the tissues of greater amounts of bacteria renders bacterial growth in the tissues easier *i. e.* increase invasiveness. This means that in infection experiments we may find that the invasiveness of a bacterium seems to augment with the infecting dose. — It is also possible that bacterial products when generated, *e. g.* in the intestinal canal, damage the epithelium of the wall and thereby facilitate infection. In a case like this we have correlation between toxicity and infectivity.

In the following these general observations will be applied to a special case: the *Salmonella typhimurium* infection in mice.

All the experiments to be described were performed with *S. typhimurium* cultures derived from one, strongly pathogenic strain isolated from a case of paratyphoid fever. From this strain a practically apathogenic variant was isolated by *Jensen* (1) after some 200 passages at gradually increasing temperature, and the courses of infections with this variant and with the original strain were examined. Examinations of all cultures showed that the infectivity of the bacteria did *not* change much when the bacteria lost their pathogenicity; this could be seen from the density of growth from different organs of newly infected mice. Following the course of infection it was found that not until bacterial foci in the tissues had been established could any difference between the strains be detected; the highly pathogenic strain was able to spread in the organism and create still deeper foci, while the apathogenic variant failed to do so. The reason for this difference between the courses taken by the *already established infections* must be that while the pathogenic strain is able to multiply and spread in spite of the antibacterial forces of the organism, the apathogenic variant succumbs to the forces acting in and around the primary foci. In other words, *the pathogenic strain shows greater invasiveness than the variant*. Remembering what has been said about a possible correlation between invasiveness and toxicity, we must examine the following two possibilities: a) The observed difference in invasiveness can be the result of a real difference in the resistance of bacteria from pathogenic and apathogenic cultures respectively to the antibacterial forces of the living organism; or b) the difference in invasiveness may be the indirect effect of a difference in toxicity between the pathogenic strain and the apathogenic variant. — We shall now examine these two possibilities:

a) The *S. typhimurium* strains and the variants are, all of them, resistant to heated serum even in high concentrations, while on the other hand they are more or less affected by fresh, normal serum. Moreover, the antibacterial effect of fresh serum can be totally suppressed by different anticomplementary agents or procedures (2,3). The different variants of *S. typhimurium* employed are thus all unaffected by thermostable bactericidal substances, such as Leucocidin, β -lysin, and the like, while they are susceptible to the action of complement. — As shown by the writer (2) there is every reason to regard complement as the active principle not only in the hemolytic and the bactericidal processes but also in the very important phagocytosis-promoting (opsonizing) action of fresh serum — an action which largely determines the degree of phagocytosis by leucocytes. — From this we may draw the conclusion that, in the case of *S. typhimurium*, a test for resistance of the bacteria to complement is essentially the same as a test for resistance to the principal antibacterial forces of the normal, non-immunized organism. A strain with great resistance must therefore be expected to be able to multiply in the organism i.e. must possess great invasiveness.

With the pathogenic strains many *in vitro* tests for resistance to complement have been made, and it has always been found that the pathogenic strain is much more resistant than the apathogenic, *whether measured in bactericidal or in phagocytosis experiments* (2). The difference in resistance to complement found in *in vitro* experiments thus agrees fully with the difference in invasiveness found in infection experiments and may alone explain this.

However, we must also consider the possibility (b) of a difference in toxicity between the strains:

b) If an animal is infected with a relatively small dose of the pathogenic strain and the course of the infection is followed, it is found that not until the bacteria have spread throughout the organism is there any sign of intoxication in the animal. The strain is thus not very toxic and depends for its pathogenic effect essentially upon the multiplication of the bacteria in the organism, i. e. upon the invasiveness. — If the infection is started by the injection of *large* amounts of bacteria intraperitoneally it is found that even the strain with low pathogenicity is able to spread and kill the animal (Table I). The toxic effect of the large amounts of culture injected is thus great enough to render multiplication of even the relatively apathogenic strain possible; and when multiplication is brought about the course of the infection is much the same as in the case of the pathogenic strain.

If the experiment is performed with varying large doses of *killed* culture + a constant, small dose of *live*, *apathogenic* culture, it is found that the toxic effect of the large doses of killed culture is sufficient to make it possible for the few apathogenic bacteria to multiply, spread and finally kill the animals. It will be seen from Table I, that

heat-killed and formol-killed cultures, of both the pathogenic and the apathogenic strain have this promoting effect on invasiveness; and, as far as the size of the experiment allows us to conclude, there is no significant difference between the effects of the 2 pairs of preparations of killed cultures.

Table 1.

Mean Death-Times in Days (t) for Mice Infected Intraperitoneally with *Salmonella Typhimurium*.

log d	live cultures		heat-killed cultures		formol-killed cultures	
	a	b	a	b	a	b
- 0.3	< 1	< 1	10.3	6.7	4.0*	12.0
- 0.6	< 1	< 1	6.5*	> 15	6.0	7.3
- 0.9	< 1	2.6	> 12	12.5*	8.3	> 15
- 1.2	< 1	4.3	> 16	∞	> 13	∞
- 1.5			> 16	∞	> 17	> 17
- 3.3	5.7	∞	constant amount of live b-cult. added (log d = -3.3)			
- 4.0	6.0**)	∞**)				
- 5.0	5.4**)					
- 6.0	14.0					
- 7.0	12.0					

*) In each group 1 mouse died within 48 hours; these early deaths are not included.

**) 5 or more mice in each group.

Culture a: pathogenic strain (= subculture 1 in Table II).

Culture b: apathogenic variant (= subculture 4 in Table II).

Log d indicates the volume (in cc.) of 20 hours old culture contained in the 0.5 cc. injected into each animal. In the experiment with killed cultures, a small, constant volume of live b-culture was added to each dilution of killed culture: $-\log d$ (live b-cult.) = -3.3. Where nothing else is stated three mice were used per dose. In cases where 1 or 2 mice survived the observation period of 20 days, t is given as $> e$, g. $1/3$ ($7 + 12 + 20$), where the figure 20 stands for a surviving animal, 100 % survival is indicated by ∞ .

In a control experiment with the same doses of killed cultures alone, a few animals died without signs of infection. Of the 16 mice receiving the 2 largest doses 3 died, whereas 22 out of 24 died in the experiment with addition of live, apathogenic culture. In this last experiment cultures from heart-blood of dead animals showed generalized infections in all cases.

Two more observations must be mentioned here: it has proved impossible to demonstrate any difference in antigenic structure between the pathogenic and the apathogenic strain. This was first showed by Kauffmann (4) and has later been confirmed by the writer and it means that the heat stable O-antigens, which constitute the main toxic substances of the bacteria, are the same in the pathogenic and the

apathogenic strain. Finally, it should be mentioned that a cell-free filtrate of a bouillon culture of *S. typhimurium* has some anticomplementary effect, and that no difference is found between the effects of filtrates of pathogenic and of apathogenic cultures; i. e. the extracellular substances of the cultures do not differ in their actions towards complement.

Summarising the statements given under a) and b) we arrive at the following reasoning: No major difference between the toxicity of the pathogenic and the apathogenic strain can be demonstrated, and the great difference between their resistance to complement may, therefore, be taken as the main cause of the difference in invasiveness. We are thus dealing with a special case where a difference in pathogenicity can be analyzed and ascribed to a difference in invasiveness. In this case, with invasiveness as the main variable, and with bacteria which are unaffected by the thermostable substances of serum, it is reasonable that we should find a positive correlation between *pathogenicity* and *resistance to complement*. Details will now be given of an experiment designed to confirm the earlier observations of the existence of such a correlation.

Experimental:

When the resistance to complement of a bacterial strain is examined the result is most conveniently given as a survival percentage, computed from an initial value, giving the number pr. unit vol. of live bacteria at the beginning of the experiment; and from a value giving the number pr. unit vol. of bacteria surviving treatment with complement. It is clear that percentages thus arrived at indicate the relative *resistances* of the entire bacterial populations. This is a very important thing to notice, because even a very low mean resistance will be found in an inhomogeneous culture containing e. g. 10 % pathogenic and resistant bacteria + 90 % apathogenic bacteria with low resistance. If this mixed culture is tested for invasiveness it may as can be seen from Table I, 1st column, show the same high invasiveness as a culture containing 100 % pathogenic bacteria, since doses of e. g. 10^{-4} and 10^{-5} cc. give the same death rate and time.

The following experiment was made with a culture which had shown the unexpected combination of relatively low resistance and full pathogenicity. In order to test the homogeneity of the culture, subcultures were made in this way: the culture was diluted to such an extent that, of 30 tubes inoculated with 0.1 ml. each only 7 showed growth. From the binominal distribution it can be calculated that, probably, not more than one of the subcultures has started from more than a single bacterium. — 6 of the 7 subcultures were used, determinations of their resistance to complement *in vitro*, and infection experiments being started simultaneously. The results are given in Table II.

The technique employed for the resistance determinations has been described in detail by the writer (2). Here it may be recalled that the experiments are carried out with cultures diluted to such an extent that the possible anticomplementary effect of dissolved bacterial products is negligible; and that in all experiments fresh, human serum is used which has been tested in a special way to make sure that it contains no immune bodies. — It may be argued that it would be more rational to use mouse serum when we want to compare pathogenicity to mice with resistance to complement. Mouse complement is, however, not stable. Mouse serum shows no complement activity because it lacks C_2 (the heat labile end-piece of complement), which, presumably is unstable in mouse blood. If C_2 , prepared from serum of another species, is added to mouse serum full complementary activity is restored (5). — A statistical evaluation of the significance of a difference between two survival percentages presents special problems, as the four figures from which the percentages are computed are independent, stochastic variables. These problems are discussed in another paper (6) and will not be dealt with here as the differences encountered in the present experiments are so great that no statistical evaluation is needed.

Besides the infection experiments and the determinations of resistance to complement a third series of experiments were made with the 6 subcultures in Table II. Using carefully washed, human leucocytes the phagocytability of the cultures was examined in a virtually complement-free milieu. The phagocytosis technique employed is described elsewhere (2), and it shall only be mentioned here, that the degrees of phagocytosis are given as percentages of free (*i. e.* unphagocytized) bacteria; and that the *live* bacteria alone are registered. The results of the tests for resistance and phagocytability are thus strictly comparable, as in both cases only the live part of the bacterial population is registered.

Table II.
Determinations of Pathogenicity, Resistance to Complement and Phagocytability in Complement-free Milieu.

one-cell subculture No.	animals surviving infection	bacteria surviving complement	free bacteria after phagocytosis*)
1	0 %	76 %	51 %
2	0 %	76 %	56 %
3	0 %	57 %	55 %
4	80 %	1 %	50 %
5	100 %	2 %	54 %
6	100 %	1 %	77 %

*) complement concentration in phagocytosis milieu less than 1 : 20,000.

Table II shows how the 6 subcultures, described above, fall out in two qualitatively different groups; cultures 1—3 showing the combination of high pathogenicity *and* great resistance, while cultures 4—6 are nearly apathogenic and exhibit low resistances. It was mentioned above that one of the subcultures might have originated from two bacteria, and if we assume such two bacteria to be of very different resistance, the resulting, mixed culture must exhibit an intermediate resistance value. Subculture no. 3 in Table II seemed to show suspicions of inhomogeneity and was, therefore, twice split up into one-cell cultures (16 in all), which, however, showed resistances lying between 43 % and 70 % and clustering around 55 %; *i. e.* there is no reason to think that culture no. 3 consists of two groups of bacteria with greatly varying mean resistances. In the one-cell cultures employed only some 40 bacterial generations have occurred in the course of between 16 and 18 hours, and it is, therefore, fairly certain that the cultures are homogeneous; *i. e.* that an estimate of resistance or phagocytability is representative of *all* the individuals in the culture*). Having taken the necessary precautions with respect to homogeneity of the cultures, we may now compare the results of the experiments in Table II with earlier experiments. As mentioned before, a series of *in vitro* experiments have been made with the original, pathogenic strain and the apathogenic variant (no 206) isolated by *Jensen* (1); some of these experiments have been described by the writer (2), and they all show the pathogenic strain to be by far the most resistant. The experiments in Table II, where 3 new, apathogenic cultures have been isolated, and where *simultaneous* infection experiments and *in vitro* determinations were made, thus fully confirm the scattered, earlier observations.

Table II includes determinations of the phagocytability of the bacteria from the 6 subcultures. These determinations show that, *when no complement is present, phagocytosis of pathogenic and apathogenic bacteria is equally strong*. The fact that *no* difference is found between the pathogenic and the apathogenic strains in phagocytosis experiments *until* complement is introduced, indirectly supports our conception of complement, and especially its phagocytosis-promoting action, as the deciding factor in the defence against the *Salmonella typhimurium* infection.

Conclusion.

Salmonella typhimurium is, normally, highly pathogenic to mice, but loss of pathogenicity occurs spontaneously, presumably through a mutative change. The relatively apathogenic variants, examined here, are characterized as follows:

*) Of course, it must be assumed that the individuals, even of a young, one-cell culture, are distributed, with respect to any given property, around a mean value. In the case of the young, one-cell culture, however, the variance of this distribution must have the lowest value attainable under experimental conditions.

- 1) They are morphologically and antigenically identical with the original strain.
- 2) They possess nearly the same infectivity and toxicity as the original strain.
- 3) Their low pathogenicity is due to reduced invasiveness.
- 4) Together with invasiveness they have lost most of their resistance to complement *in vitro*.

Part II.

In the first part of this paper the permanent, mutative change in pathogenicity and resistance to complement has been dealt with. — Change of resistance can, however, also take place in another way: — Under poor growth conditions, cultures with low mean resistances are often formed, but in this case the original, high resistance is restored on transfer to a richer medium. The fact that both loss and return of resistance can be artificially induced makes it possible to investigate the metabolic mechanism governing the development of the normal, high resistance. — Of course, it can not, at present, be decided whether the *permanent* loss of resistance, so closely related to loss of pathogenicity, and the *temporary* loss now to be dealt with, are caused by disturbances in the same mechanism; however, the mechanism of the temporary loss *can* be investigated, and a careful study of this mechanism may give us the means by which later, the problem of the permanent loss of resistance can be approached.

It should be pointed out at once that no qualitative change in pathogenicity accompanies the temporary loss of resistance, which only leads to a slight increase in the mean deathtime. It has not been possible to decide whether this is due to heterogeneity of the cultures, which in spite of their low mean resistances may contain a small fraction of bacteria with unimpaired pathogenicity and resistance; or if some of the sensitive bacteria regain resistance and pathogenicity inside the infected organism. (The writer has shown that, possibly, bacteria injected into an organism will have time to change their properties before the complement process is activated (7)). An example will show the relatively high pathogenicity of a culture with temporarily reduced resistance: Of the bacteria in the culture only 3 % survived treatment with complement, yet 18 out of 20 mice were killed the mean death-time being 9 days as compared with 100 % mortality and a mean death-time of about 6 days in a control experiment with a fully resistant culture. If the high pathogenicity of the very sensitive culture from this experiment is compared with the very low pathogenicity of the corresponding sensitive cultures (4—6) in Table II, it is seen how important it is to distinguish between temporary and permanent loss of resistance.

A curious observation led to the studies of induced, temporary

change in resistance. — If a resistant strain is passed from a culture in ordinary bouillon on to an agar plate, prepared with the same bouillon, it is found that the bacteria formed on the agar surface are less resistant than those grown in the liquid bouillon. The loss of resistance occurring during the growth on the surface is, undoubtedly, due to a *local* deficiency in the supply of one or more food-substances, arising during the growth of the colony, whose piled-up members depend on material diffusing to and through the colony. This interpretation of the phenomenon was confirmed by the subsequent observation that addition of glucose to the agar could prevent the fall in resistance. From these experiments the following, general conclusion may be arrived at: Abundant growth on an agar surface does not necessarily imply that the bacteria do not suffer a *partial* undernourishment which would not be felt in a liquid medium *with the same concentrations of nutrition-substances and growth factors*. Experiments with induced change of resistance to complement will now be described.

Experimental:

The bouillon (broth) used throughout these experiments is prepared as follows: Finely minced beef muscle is extracted overnight with its own volume of water in the cold. After boiling for 10 min. the water is pressed off and the meat once more boiled with the same volume of water. The two lots of watery extract are mixed and 1 % Riedel peptone, 0.3 % NaCl and 0.2 % Na_2HPO_4 , 12 H_2O are added. This bouillon contains: Total N (Kjeldal) about 3 mg/cc.: α -amino N (v. Slyke) about 0.4 mg/cc. and about 0.4 ‰ of fermentative, reducing substances (chiefly glucose).

Experiments were first made with bacteria grown in diluted bouillon, and, as shown in Table III, dilution beyond a critical step results in the formation of bacteria with lowered resistance. In the three experiments in Table III, the different tubes with diluted bouillon were inoculated each with about 2×10^3 bacteria, transferred from a grown out bouillon culture, which means that extracellular compounds in the bouillon are diluted about 1,000,000 times. The determinations of growth and resistance were made between 18 and 20 hours later *i. e.* several hours after growth had stopped and at a time when nearly all bacteria must be in the resting-phase. The critical bouillon concentration, below which resistance falls, is somewhere about 1/64. At the bottom of Table III are stated the values obtained from a culture in bouillon 1/256 + glucose 0.02 %, and it is seen that while *glucose raises resistance* above the original value, no increase in output is found. As the strains employed can utilize glucose as their only carbon-source, this last observation indicates that the growth-limiting factor in diluted bouillon is the concentration of N-sources.

Table III.

Determinations of Output and Resistance to Complement of Bacteria Grown in Diluted Bouillon.

bouillon conc. (c)	live bacteria pr. cc ($\times 10^{-7}$), (= n)			relative output n/c ($\times 10^{-2}$)			bacteria surviving complement		
	experiment no.			experiment no.			experiment no.		
	1	2	3	1	2	3	1	2	3
1/4	—	103	128	—	4.1	5.1	—	56%	67%
1/8	55	52	56	4.4	4.2	4.5	61%	72%	73%
1/16	32	30	27	5.1	4.8	4.3	79%	73%	70%
1/32	15	17	14	4.8	5.4	4.5	85%	60%	71%
1/64	9.3	17	17	6.0	10.9	10.9	64%	42%	51%
1/128	6.0	6.2	8.5	7.7	7.9	10.9	30%	29%	21%
1/256	—	4.7	4.0	—	12.0	10.2	—	27%	27%
1/512	—	2.9	—	—	14.9	—	—	23%	—
1/256 + glucose 0.02%	—	—	3.7	—	—	9.5	—	—	87%

Bouillon dilutions made with 0.9% NaCl in dest. water and incubated at 100° for 10–15 min. — 5 cc. in each tube; inoculation at room temp. with one drop of a dilution 1/10,000 of a fresh culture in full bouillon.

It was next ascertained that the growth proper is not influenced by dilution of the bouillon; in dilution 1/200 the values for lag phase and generation time were of the same order as in dilution 1/4. It was, however, found that the ratio: bacteria formed/conc. of bouillon, (the ratio n/c in Table III) increased definitely with dilution. It has not been ascertained whether this is due to a relative increase in the total mass of bacteria formed, or if the death-rate in grown out cultures is lower in diluted than in undiluted bouillon (where a higher concentration of toxic products will be reached). — The figures in Table III point to a possible correlation between fall in resistance and increase in relative output; this is, however, not a constant finding, and the relative increase may continue after minimum resistance has been attained. — As to the time of examination of the cultures, this is not very critical. In the interval between 18 and 22 hours after inoculation in bouillon, diluted 1/64, resistance did not vary significantly, and the number of live bacteria per cc. fell from 14 only to 10×10^7 .

Determinations of pH in the grown out cultures indicate that the drop in resistance occurs when, during growth, pH in the medium falls to values \leq about 6.2. As mentioned above resistance only drops when growth takes place in pure diluted bouillon; when *e. g.* glucose is added in sufficient concentration no drop occurs in spite of the fact that pH (outside the cells!) falls to values as low as 5.6 to 5.8. Control ex-

periments with bouillon diluted in phosphate buffers of varying pH have shown that in the interval 6.5—7.5 no drop in resistance occurs while pH-values below about 6.0 give bacteria with uniformly low resistances.

Not all cultures behave alike on transfer to bouillon diluted in saline. In some cases only minor drops in resistance are found, an observation which may be explained if we assume that in such cases pH falls less than usual. If cultures in diluted bouillon shall exhibit the characteristic low resistance we must, therefore, inoculate from a suitable culture, or dilute the bouillon in phosphate buffer with pH between 5.5 and 6.0. Prepared in one of these ways cultures in diluted bouillon can be used for determinations of the influence of different compounds on the development of resistance; this assay method is illustrated in Table III, where the effect of addition of glucose is tested, (compare the two last figures in the last column).

Based on this method the common carbohydrates, some polyvalent alcohols and C3 and C4 acids have been examined. Among these compounds, glucose was most extensively used and it was found that addition of above 0.0002 % ($= 10^{-5}$ molar) to a bouillon diluted 1/200 made itself clearly felt in the resistance of the bacteria grown in the bouillon; *i. e.*, the *fall* in resistance normally found in the diluted bouillon was significantly *diminished*. The amount of glucose mentioned (0.0002 %) means approximately a doubling of the concentration of glucose in bouillon diluted 1/200. — As it was not possible to titrate the effects of all compounds, a concentration of 0.01 %, which in the case of glucose is 4—5 times what is needed for maximal effect, was used in a series of qualitative experiments. In Table IV the results of these experiments are listed together with the results of growth experiments, in each of which one of the compounds was used as the only carbon source (the substrates composed of $(\text{NH}_4)_2\text{SO}_4$, Mg SO_4 and phosphate buffer plus the carbon source). The effects on resistance are classed in four groups, *viz.* no effect ($\gg 0\ll$), uncertain effect ($\gg 1\ll$), certain effect ($\gg 2\ll$), and strong effect ($\gg 3\ll$). As an example of a strong effect see experiment 3 in Table III, where the resistances with and without addition of glucose are 87 and 27 % respectively; on the same percentage level an effect of *e. g.* 55 % ctr. 27 % would be described as certain ($\gg 2\ll$), and as uncertain ($\gg 1\ll$) would be classed an effect of *e. g.* 40 % ctr. 27 %. Most of the compounds listed in Table IV have been used in two or more independant experiments the results of which have always agreed; fluctuations between effects $\gg 0\ll$ and $\gg 1\ll$ or between $\gg 2\ll$ and $\gg 3\ll$ have occasionally been found, but a compound once giving a «certain» effect has never on repetition given an «uncertain» effect and *vice versa*. Compounds only tested once are marked out in Table IV with an asterisk.

Table IV.

The Effects of Some Compounds on the Resistance of Bacteria Grown in Bouillon Diluted 1:200, and Growth Experiments with the Same Compounds as Carbon Sources.

Name of compound	Effect on resistance	Visible growth after:	
		30 hours	52 hours
d-glucose	3	1—1	3—3
d-mannose')	3	2—2	3—3
d-galactose	3	2—2	3—3
d-fructose	3	0—0	3—3
sorbitol')	3	3—3	3—3
mannitol')	2	3—3	3—3
dulcitol	1	0—0	3—3
arabinose	2	1—1	3—3
d-ribose	3	0—0	0—0
xylose	0	0—0	0—1
erythritol	0	0—0	0—0
glycerol	3	1—1	3—3
Na-citrate	3	1—1	2—2
Na.succinate')	3	0—1	2—2
Na-tartrate	3	0—0	0—0
Na-malate')	3	2—2	3—3
Na-fumarate')	3	0—1	2—2
Na-maleinate	1	0—0	0—0
Na-pyruvate	1	1—1	2—2
Na-lactate	1	1—1	3—3

The effects on resistance are classed according to strength; »0« meaning no effect and »3« indicating max., positive effect; no negative effects were observed. Compounds marked with *) have only been examined once for effect on resistance, (see text). The growth experiments were carried out twice, in some cases 3—4 times; concentrations of C.compounds 0.2 %; inoculations made each with about 500 bacteria from a culture in undiluted bouillon; no visible growth indicated by »0«; max. turbidity, corresponding to about 10^9 bact./cc., indicated by »3«. — In all cases some growth could be demonstrated, but in the cases marked »0«, it amounted to only about 8—10 divisions in 72 hours, and no turbidity could be observed.

Summarizing the findings in Table IV we see that the common hexoses all give strong, reproducible effects. The pentoses examined are not uniformly effective: d-ribose, arabinose and xylose giving the reactions 3, 2 and 0 respectively. The same is the case with the polyvalent alcohols: sorbitol, mannitol and dulcitol giving reactions 2, 2 and 1, erythritol giving reaction 0. Glycerol and citrate are both very active, and so are the C₄, dicarboxyl acids, except maleic acid which has only a minor effect. Pyruvic and lactic acid have only slight effect. — In no

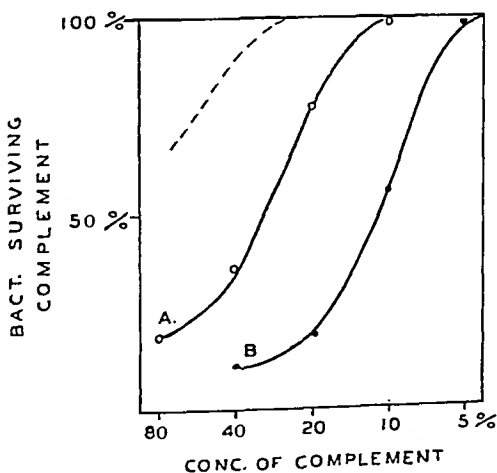
case was a certain resistance-diminishing effect observed. The effects of the compounds on resistance and their utilizability as carbon-sources are seen not to run parallel in all cases. *E.g.* d-ribose and tartrate has a strong positive effect on resistance and give only slow and scanty growth, while lactate has slight effect on resistance and gives good growth.

The principal feature of the experiments just described is the observation that under poor growth-conditions the process on which the development of resistance depends is slowed down and gets out of step with the growth proper. This means that under such conditions bacterial division will occur before the bacterium has had time to synthesize its normal amount of some, so far unidentified, substance on which its resistance depends. — The effect of glucose and the other positively reacting compounds may best be described as an acceleration of the synthesis of the unknown substance.

The effect of glucose and related compounds on the resistance of bacteria grown in diluted bouillon or on an agar surface made it desirable to see if phosphorylation is involved in the process on which the development of resistance depends. — It was found that phloridzin could be added to bouillon without seriously affecting the growth of *S. typhimurium*. The specific inhibitory effect on the uptake of inorganic phosphorus exercised by phloridzin could thus be investigated in a growing culture. In Table V a titration experiment is shown in

Table V.

Titration of the Effect of Complement on Bacteria Grown with and without Phloridzin in the Medium.



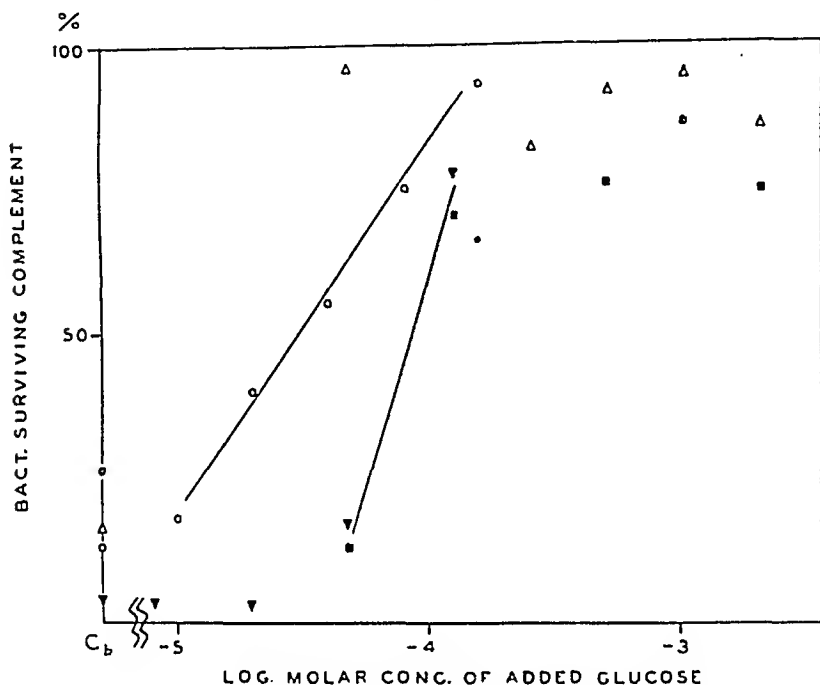
The dotted, upper curve indicates the result that would be obtained with bacteria grown in undiluted bouillon.

Curve A shows results with bacteria grown in bouillon 1/200.

Curve B shows results with bacteria grown in bouillon 1/200 + phloridzin (m/200).

which the resistances of two cultures are determined at different complement concentrations: The upper, dotted curve, inserted for comparison, roughly indicates the great resistance of bacteria grown in undiluted bouillon; curve A pictures the considerably reduced resistance of bacteria grown in bouillon diluted 1/200, and curve B shows the further reduced resistance of bacteria grown in bouillon 1/200 to which is added phloridzin in a concentration of m/200. Titration experiments like this should be made in all cases where the sensitivity of the bacteria makes it possible to examine the greater part of the curves, in order to make sure that the effect studied concerns all

Table VI.
The Effect of Phloridzin on the Resistance to Complement at Varying Concentrations of Glucose.



The signs in the Table indicate survival percentages from different experiments; each experiment has its own sign, and open signs (e. g. Δ) refer to cultures *without* phloridzin while filled out signs (e. g. \blacktriangle) refer to cultures with addition of phloridzin (m/200).

c_b indicates the concentration of glucose in bouillon diluted 1/200, i. e., about 10^{-5} molar.

Using a method of computation suggested by Rash (6), it can be shown that the 6 percentages derived from tubes *without* phloridzin and with glucose-conc. above 10^{-4} molar, do not differ more than is to be expected if they are assumed to belong to one group with the mean value 89 %. — The 5 percentages from tubes *with* phloridzin and with high glucose-conc. can also be regarded as belonging to one group (mean value 72 %). The mean values of the two groups described differ significantly.

bacteria in the culture. If *e. g.* the decrease in resistance caused by phloridzin had affected only a fraction of the bacteria, the titration curves would *not* have shown parallel courses. The parallelism between the curves in Table V indicates that, as far as the process affected by phloridzin is concerned, no qualitative difference exists between the bacteria in the culture.*)

Finally, we shall compare the opposite effects on resistance of glucose and phloridzin respectively. In table VI are collected various determinations of the resistances of bacteria from cultures with, and without phloridzin and with varying concentrations of glucose. In this Table each value indicates the resistance of a culture, all cultures having been tested with the same concentration of complement (*viz.* 50 %). It is seen that though some variation is found between corresponding results from different experiments, there is no doubt that the effect of phloridzin is strongest at low concentrations of glucose and increasing glucose concentration diminishes the effect, (compare the phloridzin effect at glucose concentrations of about $10^{-4.5}$ molar to the effect at concentrations above 10^{-4} molar). This seems to indicate that phloridzin interferes with the utilization of glucose in the building up of resistance.

It should be mentioned that phloridzin concentrations of about $m/200$ besides a decrease in resistance bring about a minor decrease in the output of bacteria. This means that phloridzin not only causes the drop in resistance to increase in the diluted bouillon but at the same time causes the growth process, during which the drop occurs, to stop earlier. If phloridzin had effected the growth process alone, a slightly smaller drop in resistance would have been expected owing to the shortening of the growth process. The effect of phloridzin on the growth of the bacteria thus does not alter our idea about its inhibiting effect on the development of resistance.

Conclusion.

A temporary (reversible) drop in the resistance of *Salmonella typhimurium* to complement is found when the bacteria are grown in bouillon diluted beyond a certain limit. This often considerable drop in resistance occurs within the relatively short period of 14—15 bacterial divisions. Addition to the diluted bouillon of glucose, or one

*) In an earlier paper the writer (2) has shown that in most cases titration curves, like those in Table V, are symmetrical, S-shaped curves which in a probit-diagram give straight lines indicating that the bacteria are normally distributed with regard to resistance to complement. It has later been found that when cultures, which, for the completion of the curves, demand higher concentrations of complement than about 30 %, are examined, an upwards concave curve is obtained in the probit-diagram. This seems to indicate that at high concentrations the increase in complement activity does no longer correspond to the increase in concentration.

of several related compounds, completes the bouillon in such a way that no drop in resistance occurs. Phloridzin which inhibits the uptake of inorganic phosphorus, counteracts the effect of glucose which thus seems to undergo phosphorylation before it is utilized. It is at present impossible to say whether glucose and the similarly reacting compounds are used directly to build up some substance which renders the bacteria more resistant; it may well be that the different compounds only supply energy for the synthesis of this substance, or they may be used by the bacteria to keep the *pH of the internal environment of the cells* within limits permitting full development of resistance (cf. p. 423—24).

Using synthetic substrates, further studies of the resistance-producing mechanism are being made; the first results of these experiments will be published shortly.

Summary.

Part I.

The conception of pathogenicity is analysed and split up in three components, *viz.* *infectivity*, *invasiveness* (here defined as the faculty for multiplication inside the invaded organism) and *toxicity* (indicating the general dangerousness of the bacteria). Possible correlations between the three factors are discussed.

Infections with pathogenic and apathogenic strains of *Salmonella typhimurium* are described, and it is shown that the difference in invasiveness between the strains must be caused mainly by a difference in their resistance to the antibacterial forces of the organism (*in casu* complement). No significant difference in toxicity is found between the strains, and morphologically and antigenically they are also identical.

In an experiment 3 new apathogenic cultures are isolated, and the homogeneity of the cultures with respect to invasiveness and resistance to complement is examined. The experiment confirms earlier observations by the writer showing that pathogenic strains are much more resistant to complement than are apathogenic strains. — It is shown that in complement-free milieu there is no difference between the phagocytability of pathogenic and apathogenic bacteria.

Part II.

As opposed to the above described *permanent*, mutative loss of pathogenity and resistance, the occurrence of a *temporary, reversible* loss of resistance is described. Temporary loss of resistance is found when *e. g.* the resistant strain is grown in diluted bouillon, and it can be prevented by adding glucose or one of several other simple, N-free compounds to the bouillon. That phosphorylation is probably involved in the resistance-producing process, is concluded from experiments with phloridzin, which is shown to lower resistance considerably. Glucose counteracts to some extent the phloridzin effect.

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PLEOMORPHISM IN *HEMOPHILUS HEMOLYTICUS* CAUSED BY V-FACTOR DEFICIENCY

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A few small transparent strongly hemolytic colonies were found in a throat culture from a woman, with a history of frequent sore throats. They consisted of gram-negative rods of a very pleomorphic appearance. Some of the microbes were long filaments with large spherical or fusiform swellings along the filament, or spermatozoa-like with a swollen, round or oval head and a long tail. A number of large free spherical bodies were also found (photo no. 1).

At the same time two similar strains were isolated from two patients in the eye clinic. The three strains were found to require the V-factor of Thjøtta and Avery, and were classified as *H. hemolyticus*.

As this species has seemed to be a rare finding lately in this laboratory, a class of medical students, numbering 44, was examined for the presence of this microbe in the throat. It was found in 8 out of the 44 cultures, but only 6 of the strains were obtained in a pure culture. All strains showed a similar, more or less marked pleomorphism.

Some experiments were undertaken to study the cause of this pleomorphism, which seemed particularly interesting because of the frequency of the much disputed »spherical bodies« or »large bodies«.

Material.

8 strains, all isolated from throat cultures, were studied. Their properties were the following:

Morphology. All strains were gram-negative rods, with a more or less extreme pleomorphism. Some strains mainly produced short rods with little pleomorphism, others produced mainly long filaments, often with large club-like swellings of one end or with large fusiform or oval swellings along the filament. Large free spherical or oval

bodies and other bizarre »involution forms« were also frequent. Pleomorphic cultures were further characterized by irregular staining. While some microbes were stained very strongly, a large number were very pale, often practically unstained, and seemed to be in a process of autolysis. The colonies were flat, smooth or slightly irregular, transparent, of a diameter up to 2 mm, and produced wide zones of hemolysis on blood agar. All strains showed an absolute need for the V-factor, in the form of blood, yeast extract or produced by some other microorganism such as a *Staphylococcus*. All strains showed a marked satellite phenomenon. The strains produced acid from glucose, maltose and sucrose but not from mannitol or lactose. They did not produce indol.

The strains conform with *Hemophilus hemolyticus* except in their independence of the X-factor (1), and were classified as atypical strains of this species. They are perhaps the same as the hemolytic *H. parainfluenzae* studied by Dienes (2).

Experimental.

In a study (3) on some mucoid organisms from the throat, a very marked pleomorphism could be produced whenever the strains were exposed to a dry atmosphere at 37 C, while it did not appear in a humid atmosphere at 37 C, or at lower temperatures. It was thought that the pleomorphism in the strains under investigation might be due to some similar factor.

Experiment no. 1.

3 strains were inoculated on two different blood agar plates, one of which was incubated in a dry atmosphere (in an ordinary electric incubator), the other in a humid atmosphere (in a tight jar containing a dish of water). After 24 and 48 hours a striking difference was found. The plate grown in a dry atmosphere showed extreme pleomorphism, the other plate practically none. Thus the hypothesis seemed to have been confirmed (photos nos. 2, 3). All attempts to reproduce the results, however, were unsuccessful. Thus it seemed as if some other difference between the two plates used in the first experiment might have been the cause of the observed difference. Nothing is known about the origin of the blood used in the first two plates, but there is a fair chance that different samples had been used in the two plates. When the same sample of blood was used in all plates, no difference was found between cultures grown in a dry or a humid atmosphere.

In this laboratory blood for blood agar plates is usually drawn from medical students or from patients in the hospital on two or three days of each week. Some samples are used immediately, others are stored with sodium citrate at 4 C until required, up to 3 or 4 days. It seemed to be possible that the different treatment of the dif-

ferent samples might be the cause of the varying degree of pleomorphism.

Experiment no. 2.

20 ml samples of blood were drawn from the same healthy individual on 5 consecutive days, and stored at 4 C with sufficient citrate to prevent coagulation. On the last day 2 samples were drawn, one of which was mixed with sodium citrate solution, whereas the other was used without anti-coagulant. Plates were prepared of all samples with the same percentage of blood. Two sets of plates were inoculated with 6 different strains and incubated in a dry and a humid atmosphere respectively.

Microscopical examination after 24 and 48 hours revealed no appreciable difference between the different cultures of the same strains. Very little pleomorphism was found in these cultures.

Neither addition of citrate nor storage seemed to have any influence on the degree of pleomorphism.

The possibility remained that some other qualitative or quantitative difference between the plates, used in the first experiment, was the explanation of the results. There might be some reason to suspect that a different quantity of V-factor might be the cause. It has been noted repeatedly in this laboratory that not all blood agar plates give equally good growth of hemophiles. In some cultures they only grow as satellites round other colonies.

Experiment no. 3.

Yeast extract, prepared according to the directions given by Thjøtta and Avery (4), was used as the source of V-factor.

Two series of three-fold dilutions of yeast extract were made, one in ascites fluid, the other in 0.85 % saline. 3 ml of each of these dilutions were mixed with 12 ml. of melted agar, and poured into Petri dishes.

The plates were inoculated with 6 different strains and incubated at 37 C in a humid atmosphere.

On control after 24 and 48 hours no difference was found between the ascites series and the saline series, but a very marked increase in pleomorphism was found to appear with diminishing concentration of yeast extract. In cultures containing the largest amount of yeast extract (2/3 ml), very little, if any, pleomorphism was found. The microbes were mostly short and homogeneous. Only the strains with the highest tendency to pleomorphism produced occasional spherical bodies in these cultures.

The next two or three dilutions of yeast extract, however, produced a very strong increase in pleomorphism, with an increase in the number of filaments, the number of fusiform and spherical swellings and free spherical bodies, and also a very marked increase in the number of microbes which were very weakly stained.

Concentrations of yeast extract below 2/81 ml to 2/243 ml gave no visible growth. When some of the inoculum was scraped off such plates and examined, a large number of very pale, probably dead microbes were found, and here and there a few short, strongly stained rods and chains, as an indication of a feeble, unsuccessful attempt to grow.

This experiment was repeated several times with slight variations in the technique. Similar results were always obtained (photos nos. 4—7).

On some plates, containing insufficient yeast extract to give growth, some contaminant colonies — mainly staphylococci — appeared. Round such colonies good growth of the hemophiles was obtained. On examination of such colonies, those growing very close to the staphylococcus were found to contain comparatively regular, homogeneous rods, whereas colonies farther away from the staphylococcus showed a very marked pleomorphism.

Experiment no. 4.

Plain agar plates or lactose agar plates were inoculated heavily in parallel streaks with two of the strains. A staphylococcus was inoculated in one streak across the plate, at right angles to the first ones.

After 48 hours colonics had appeared in a 1—1.5 cm wide belt on both sides of the staphylococcus. The colonies close to the staphylococcus showed very little or moderate pleomorphism, while the tiny ones in the periphery were found to be extremely pleomorphic (photos nos. 8, 9).

Thus the influence of the lack of V-factor was effectively counteracted by the presence of microbes known to produce this factor.

The experiments prove that pleomorphism in our strains was produced by a deficiency of V-factor. It seems likely that a relative deficiency of this factor may also be the cause of pleomorphism on some blood agar plates.

Discussion.

Pleomorphism of the type described above is not rare in bacteria. We have repeatedly found strains of *E. coli* in cases of chronic urinary infection, which showed a similar pleomorphism. Dienes has already drawn attention to this fact (5).

Production of large swollen cells, filaments with fusiform swellings or clubs and free spherical bodies occurs occasionally in a number of species, such as *P. pestis*, *H. duplex*, *Streptobacillus moniliformis* and *B. funduliformis*. It has been considered so typical of some species as to be utilized in systems of classification (e. g. Prevot's Sphaerophoraceae (6)).

There has been a great deal of speculation as to the nature of this pleomorphism, and particularly of the role of the spherical bodies.

One school of thought, represented by Dienes (2, 5, 7), considers the spherical bodies as stages in a special kind of bacterial variation, connected with the appearance of L type colonies. Dienes has done a large amount of work to clarify this problem, and has given accurate descriptions of such phenomena in *Streptobacillus moniliformis*, *H. influenzae*, *E. coli*, gram-positive spore-bearing rods and *Proteus*.

Others, particularly Klieneberger (8), explain some instances of such pleomorphism as the result of symbiotic growth between a bacterium and an organism of the pleuro-pneumonia group.

Still others consider such phenomena as »involution forms«, caused by the action of some injurious factor, and attach no particular importance to the spherical bodies.

In a paper which has just appeared, Ørskov (9) supports this opinion and maintains that the term »involution forms« gives the most fitting description of the phenomenon.

It seems that the result of our experiments would lead us to agree with Ørskov. Both in this study and another study (3), mentioned above, a single injurious factor was found to be responsible for the appearance of pleomorphism, and it appeared in the most marked form at a point, where the injurious factor was just on the point of preventing growth altogether.

If production of spherical bodies and similar forms can be shown in each instance to occur under the influence of factors which prevent the normal multiplication and, particularly, the division of the cells, the presence of spherical bodies would lose significance as far as classification is concerned, and speculation as to their role in some hypothetical life cycle would probably be invalid.

It would seem that a useful approach to the problem would be to find out under which circumstances pleomorphism might be brought to disappear. Such studies might lead to information about the growth requirements of various species, which knowledge might be more useful in classification than the registration of abnormal cell forms.

Summary.

Several strains, classified as atypical strains of *Hemophilus hemolyticus*, were found to show a marked pleomorphism on certain blood agar plates. The pleomorphism was characterized by elongation of the cells and the appearance of fusiform swellings, spermatozoa-like cells and free spherical bodies.

Pleomorphism could be produced at will by reducing the quantity of V-factor in the media. The presence of microbes, such as staphylococci, in the cultures counteracted the lack of V-factor.

It is believed that this type of pleomorphism is due to interference with the mechanism of cellular division by some adverse influence or nutritional deficiency.

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Photo no. 1.

H. hemolyticus. Primary culture on blood agar. Many fusiform and club-like swellings. Stained with carbol fuchsin. $\times 1100$.



Photo no. 2.

Blood agar culture, incubated in humid atmosphere. (exp. no. 1). Carbol fuchsin. $\times 1850$.



Photo no 3.

Same strain as in no. 2. Dry atmosphere. Carbol fuchsin. $\times 1850$.



Photo no. 4.

H. hemolyticus grown on agar plate with 1 ml yeast extract. Carbol fuchsin. $\times 1850$.



Photo no. 5.

Same strain grown on agar plate with 0,5 ml yeast extract. Carbol fuchsine. $\times 1850$.



Photo no. 6.

Same strain grown on agar plate with 0,125 ml yeast extract. Carbol fuchsine. $\times 1850$.



Photo no. 7.

Same strain grown on agar plate with 0,0625 ml yeast extract. Carbol fuchsine. $\times 1850$.



Photo no. 8.

Satellite colonies growing close to *Staphylococcus* colony on plain agar plate. 48 hours. The microbes are weakly stained, as usual in 48 hour cultures, but show only moderate pleomorphism. Carbol fuchsine. $\times 1100$.



Photo no. 9.

Satellite colonies of same strain, growing farther away from *Staphylococcus*. Marked pleomorphism with coarse filaments and spherical bodies. Carbol fuchsine. $\times 1100$.

THE INFLUENCE ON THE FORMATION OF ANTITOXIN EXERCISED BY DEAD DIPHTHERIA BACILLI ADDED TO DIPHTHERIA TOXOID IN THE IMMUNIZATION OF RABBITS AND HORSES

By Arne Lithander.

(Received for publication November 10th, 1947).

The methods used in the immunization of animals for the production of antitoxic sera against tetanus and diphtheria were considerably improved when *Ramon* (1925) suggested that certain non-specific preparations, such as tapioca, calcium chloride and alum, be added to the antigen. *Ramon* found that horses immunized against diphtheria by diphtheria toxoid and tapioca yielded a serum containing an average of 700 A. U. per cc while when only diphtheria toxoid was used the average was less than 375 A. U. *Glenny*, *Bottle* and *Stevens* (1931) were of opinion that the value of the addition of these non-specific preparations lay in the retardation of the resorption of the antigen so that its immunizing effect was enhanced. *Ramon* (1938) thought it probable that such an addition caused a non-specific stimulation which was favourable for the immunization. *Lemétayer* (1933) pointed out that when tapioca was added to injections of diphtheria toxoid into horses, a leucocytosis developed which he considered important for the formation of antibodies.

Tapioca and alum are probably the preparations most commonly used in the immunization of horses for the production of diphtheria serum. The addition of tapioca to the antigen contributes to a good yield of antitoxin but unfortunately it causes large abscesses to form around the places of injection. This affects the horses to such an extent that the immunization must not seldom be interrupted. The addition of alum causes less formation of abscesses but is considered to give poorer formation of antibodies in horses than tapioca, *Ramon*, *Lemétayer*, *Richou* (1934). These authors reject the use of alum in horses also for the reason that the horses are seriously injured by the

preparation, *inter alia* in the kidneys. On the other hand, English serologists have found alum to be of great value and, generally speaking, without disadvantages in the production of diphtheria serum on horses.

Experiments have also been made to use diphtheria bacilli in various manners in the immunization against diphtheria. It may, for instance, be mentioned that *Böhme* (1924) inoculated live diphtheria bacilli into the skin of guinea pigs, finding afterwards that the serum of the guinea pigs contained 5—10 A. U. per cc. *Ramon* and *Debré* (1931) injected live diphtheria bacilli subcutaneously on horses. After two injections of small quantities of bacilli the anti-body content of the horses increased to 3—15 A. U. per cc. By repeated injections of larger amounts of live bacilli *Legroux*, *Ramon*, *Debré* and *Thirolloix* (1931) obtained on two horses 35 and 45 A. U. respectively. In experiments on guinea pigs *Kritschewski* and *Galanova* (1936) found that injections of dead diphtheria bacilli yielded only an average of 0.005 A. U. per cc. *D'Antona* and *Valensin* (1936) detoxicated diphtheria cultures by formalin in heat. The finished preparation, which contained 10—15.000 millions of bacilli per cc, was compared in regard to its immunizing ability with anatoxin free from bacilli. The authors found that the animals were little affected by this bacillus containing preparation and that it caused better formation of anti-bodies than a bacillus-free toxoid. However, they did not state what antitoxin values had been obtained. They regarded the effect of the bacilli as non-specific as is the effect of tapioca.

In the State Bacteriological Laboratory tapioca has chiefly been employed as an addition to diphtheria toxoid in the production of diphtheria serum on horses. Alum and calcium chloride have yielded less satisfactory results. During the war it proved impossible to obtain tapioca, so a demand arose for another suitable preparation to be added to the diphtheria antigen. In his endeavours to find one, the author mainly concentrated on the influence of dead diphtheria bacilli.

Preliminary experiments were carried out on rabbits, divided into four groups of 10 animals each. All the rabbits showed a content of normal anti-bodies against diphtheria lower than 0,003 A. U. per cc. They were given subcutaneous injections of the same diphtheria toxoid, produced from toxin prepared on Loiseau-Philippe substrate with strain Park-William. The injections were made with four days' intervals, each rabbit receiving 6, 15, 30, 60 and 240 Fl. U. of toxoid. In the first group 1 % tapioca was added to the toxoid, in the second formalin-killed diphtheria bacilli to such an amount that the toxoid contained 2 % of moist bacilli. In the third group 2 % of dead typhoid bacilli were added to imitate »vaccination associée« according to *Ramon* and *Zoeller* (1926) and in the fourth group toxoid alone was injected. Six days after the last injection of toxoid the concentration of diphtheria antitoxin in the rabbit sera was examined by intracutaneous

tests on rabbits according to *Jensen* (1933). The results are shown in Table 1.

Table 1.

Concentration of diphtheria antitoxin in serum from rabbits injected with diphtheria toxoid with the addition of various substances. n = number of rabbits, M = mean value, Min. = minimum, Max. = maximum content of antitoxin in the group.

Preparation injected	A. U./cc			
	n	M	Min.	Max.
Diphtheria toxoid + 2 % diphtheria bacilli	10	7,9	2,5	15
Diphtheria toxoid + 1 % tapioca	10	6,7	2,5	12
Diphtheria toxoid + 2 % typhoid bacilli	9	4,6	2,0	7
Diphtheria toxoid	10	2,5	0,5	4

Though the number of animals is limited the table nevertheless shows that the addition of dead diphtheria bacilli to diphtheria toxoid in the immunization of rabbits according to the method described above causes a considerably better formation of antitoxin than that obtained by injections of diphtheria toxoid alone, and the result is at least as good as when tapioca is added. The addition of typhoid bacilli appears to give a slightly improved effect as compared with the use of diphtheria toxoid alone.

The experience thus gained was applied to the immunization of horses for the production of diphtheria antitoxin. The horses were immunized in couples, so that one horse received toxoid + 1 % tapioca and the other the same toxoid + 2 % diphtheria bacilli. The toxoid held approximately 30 flocculation units per cc. As in the experiments on rabbits the diphtheria bacilli were killed by treatment with 2 % formalin at room temperature for 24 hours, after which the formalin was removed by washing with physiological saline. Fifteen couples of horses were treated. In each couple both the horses had the same content of normal anti-bodies in the serum. The content of normal anti-bodies varied between 0,003 and 0,1 A. U. per cc.

In the first period the immunization comprised 6 injections, 20, 50, 100, 150, 250 and 300 cc of toxoid. In each subsequent period were given, as a rule, three injections of 100, 150 and 200 cc. The injections were made every third or fourth day. Bleeding was done 4—5 days after the last injection. Between a bleeding and the following period of immunization the horses rested for 3 weeks. The content of antitoxin in the sera of the horses was determined by flocculation. The results are shown in Table 2. The addition of diphtheria bacilli caused considerably less formation of abscesses and had less effect on the general condition than what was noticed after the addition of tapioca. The rise in temperature after each injection was about the same in both cases.

Table 2.

Concentration of antitoxin in serum from horses immunized with diphtheria toxoid with the addition of diphtheria bacilli or tapioca. n = number of horses, M = mean value, Min. = minimum, Max. = maximum content of antitoxin in the group.

	Diphtheria toxoid + 2% diphtheria bacilli				Diphtheria toxoid + 1% tapioca			
	A. U./cc				A. U./cc			
	n	M	Min.	Max.	n	M	Min.	Max.
1st period	15	785	175	1620	15	731	156	1296
2nd »	14	637	209	1125	14	702	122	1620
3rd »	10	811	432	1400	13	856	204	1620
4th »	9	822	622	1620	9	693	373	1620

In so far as conclusions can be drawn from so scanty a material the table shows that the addition of dead diphtheria bacilli to diphtheria toxoid in the immunization of horses would appear to cause as good a formation of antitoxin as the addition of tapioca. The small number of animals prevents conclusions to be drawn from the difference between the two groups in the second and fourth periods of immunization. It is evident from the table that the number of horses decreased during the last periods of immunization. This is explained by the fact that the horses which could not resist the injections sufficiently well were excluded. This, of course, renders a comparison of the two groups more difficult. It may be mentioned that by immunization with equal doses of equivalent diphtheria toxoid without addition the mean value for the content of antitoxin was less than 300 A. U. In regard to the time of flocculation and the agreement between flocculation values and the values obtained in titrations on guinea pigs no difference could be demonstrated between »tapioca serum« and »bacillus serum«.

Summary.

In the immunization of rabbits and horses for the production of diphtheria serum the addition of dead diphtheria bacilli to diphtheria toxoid gives as good a formation of antitoxin as the addition of tapioca.

The addition of diphtheria bacilli caused in horses less formation of abscesses and less effect on the general condition than did the addition of tapioca.

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EFFECT OF PENICILLIN ON CERTAIN INTESTINAL ROD-SHAPED BACTERIA. PENICILLINASE PRODUCTION IN *PROTEUS MORGANI*

By O. Lahelle.

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The effect of penicillin on a number of different microbes and viruses has been investigated. It is naturally first and foremost the penicillin-sensitive microbes that have been subjected to the most frequent and thorough investigation, while the effect on the microbes which are generally regarded as resistant to penicillin has been tested to far less extent.

To this latter group belong the intestinal rod-shaped bacteria, some particular species of which shall be dealt with in the present paper. Already in 1929 *A. Fleming* found that penicillin had relatively little effect on *Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris*. *E. P. Abraham and coworkers* in 1941 were able to confirm the results of his investigations. They have later been extended to embrace all the ordinary pathogenic and non-pathogenic intestinal bacteria belonging to the Enterobacteriaceae, and it has been regularly found that these bacteria are resistant to penicillin.

The work done on this field in recent years has shown, however, that even though the intestinal rod-shaped bacteria must from a practical standpoint be regarded as resistant, this does not mean that they are absolutely unaffected by penicillin. But the concentrations of the antibiotic which inhibit the growth of the bacteria are so strong that in case of infections in man we cannot expect any effect from the usual subcutaneous, intramuscular or intravenous administration of penicillin. *G. L. Hobby* (1944) tested a number of Gram-negative, intestinal bacteria and found that typhoid and dysentery bacteria were relatively sensitive. *A. R. Thomas* and *Max Levine* (1945) worked with different *Salmonella* and *Shigella* types, as well as strains of the *Escherichia coli* and *Aerobacter aerogens*, and showed that all of

gan's bacilli, or the coliform microbes, as we here name them, had no power of fermenting 1-phenylalanine with formation of phenylpyruvic acid. For distinguishing between *Proteus morgani* and the coliform bacteria we used, in addition to fermentation, the different powers of decomposing urea possessed by the bacteria. While *Proteus vulgaris* and *Proteus morgani* decompose urea with great energy, the coliform bacteria do not hydrolyze that substance, or hydrolyze only small quantities thereof. We employed the technique devised by W. Blake Christensen (1946).

The titration of the sensitivity of the different intestinal bacteria to penicillin was effected by a serial dilution method, mostly a modification of Kolmer's method. Bouillon-dilutions of penicillin are here arranged in geometric progression. To each tube containing $\frac{1}{2}$ ml of penicillin-bouillon is then added the microbe to be tested. As inoculum we used a 6-hour culture in ordinary bouillon diluted 1/1 000 000. Of this dilution $\frac{1}{2}$ ml was added to each tube. Then followed incubation for 20 hours at 37° C. After incubation the degree of turbidity in the media was noted macroscopically. For control were made cultures on agar plates from the tubes in order to obtain an idea of the number of living microbes present in each tube. As a rule there was good concordance between the macroscopic readings and the readings after culture on agar. The *Proteus vulgaris* gave swarming colonies after culture on agar, and for this bacterium only macroscopic readings from the tubes were taken. The results of our titrations will best be seen from Table I.

Table 1.
Penicillin-sensitivity in the different intestinal bacteria.

	Number of strains	Penicillin concentration										Control
		2000	1000	500	250	125	63	32	16	8	4	0
<i>Proteus vulg.</i>	10						1	2	2	4		
<i>Proteus morg.</i>	10	6	2	2								
Coliform bact.	10				2	2	4	2				
<i>Escherichia coli</i>	23	1			2	1	15	4				

The material gives quite a good idea of the penicillin-sensitivity of the different bacteria. It appears that *Proteus vulgaris* is relatively sensitive. The most resistant of these strains were completely inhibited in their growth by 63 units of penicillin per ml. The other strains

had a penicillin resistance which lay between 32 and 8 units. Most of them had a resistance of 16 or 8 units per ml.

Proteus morgani proved to be very resistant to penicillin, 6 strains having a resistance of 2000 units or more. None of the strains investigated had a resistance of under 500 units per ml. The coliform microbes, or those formerly called Morgan's bacilli Types II, III and IV, occupied an intermediate position. The same was the case with strains of the *Escherichia coli*. These two groups of bacteria had a resistance lying between 250 and 32 units per ml. One of the coli strains proper, however, showed a resistance of 2000 units. The investigation shows that all the Gram-negative intestinal bacteria are sensitive to penicillin, but there is a distinct difference in resistance between the individual species.

It has been mentioned that the titration of the resistance to penicillin was effected by adding to each tube of penicillin-bouillon a 1:1 000 000 dilution of an original 6-hour culture. Now it has previously been demonstrated that the amount of the inoculum may be of importance for the result of such titrations as have here been made. This has been found by *R. F. Parker* (1946) to be the case with the more resistant, penicillinase-producing staphylococci. The same observation was made by *K. Riemers Eriksen* (1946) as regards the *Bacillus anthracis* and a single paracolon strain. We investigated many of our strains by making a series of titrations of penicillin resistance with use of different inocula for one and the same strain. Examples of these titrations are given in Tables II, III and IV.

From Table II it is distinctly evident that the amount of the inoculum is of great importance in titration of the penicillin resistance in case of *Proteus morgani*. There a 1/10 dilution of the original bouillon is used, the resistance to penicillin is more than 2500 units per ml. On use of an inoculum with a dilution corresponding to 1/10 000 000 of the original bouillon culture we come down to a penicillin resistance figure of between 312 and 150 units per ml. The titration of the *Proteus vulgaris* gave a different result. Here the amount of the inoculum seemed to be without significance for the determination of the resistance. It would perhaps be natural to ascribe this difference in the result to the varying ability of the microbes to produce penicillinase or penicillin-inactivator. As regards the staphylococci *S. E. Luria* (1946) claims to have shown that the amount of the inoculum is of significance for the titration of penicillin resistance when the strains form penicillinase, but is of no importance in case of the strains which do not produce penicillinase. The difference in the results of our titrations should therefore be due the production of large quantities of penicillinase by *Proteus morgani*, while *Proteus vulgaris* has no such power of producing a penicillin-inactivating enzyme. As already mentioned, *Amedeo Bondi* and *Catherine Dietz* (1944) found that *B. paracoli* produces penicillinase, while *Proteus vulgaris* has not that

Table 2.
Titration of penicillin resistance with different inocula. *Proteus morgani*.

Dilution of bouillon culture	Penicillin concentration									
	2500	1250	625	312	150	75	32	16	8	0
1/10	++	++	++	++	++	++	++	++	++	++
1/100	—	++	++	++	++	++	++	++	++	++
1/1000	—	—	++	++	++	++	++	++	++	++
1/10000	—	—	+	++	++	++	++	++	++	++
1/100000	—	—	—	+	++	++	++	++	++	++
1/1000000	—	—	—	+	++	++	++	++	++	++
1/10000000	—	—	—	—	+	++	++	++	++	++

Table 3.
Titration of penicillin resistance with different inocula. *Escherichia coli*.

Dilution of bouillon culture	Penicillin concentration									
	1000	500	250	125	63	32	16	8	4	0
1/10	—	—	—	—	—	++	++	++	++	++
1/100	—	—	—	—	—	++	++	++	++	++
1/1000	—	—	—	—	—	++	++	++	++	++
1/10000	—	—	—	—	—	+	++	++	++	++
1/100000	—	—	—	—	—	+	++	++	++	++
1/1000000	—	—	—	—	—	++	++	++	++	++
1/10000000	—	—	—	—	—	+	++	++	++	++

Table 4.

Titration of penicillin resistance with different inocula. Proteus vulgaris.

Dilution of bouillon culture	Penicillin concentration									
	1000	500	250	125	63	32	16	8	4	0
1/10	—	—	—	—	—	—	—	+	++	++
1/100	—	—	—	—	—	—	—	++	++	++
1/1000	—	—	—	—	—	—	—	++	++	++
1/10000	—	—	—	—	—	—	—	++	++	++
1/100000	—	—	—	—	—	—	—	+	++	++
1/1000000	—	—	—	—	—	—	—	+	++	++
1/10000000	—	—	—	—	—	—	+	++	++	++

property. We have been able to confirm this observation. Meanwhile, if we examine the results of titration of *Escherichia coli* it is seen it gives equally high degrees of resistance to penicillin, irrespective of the quantity of inoculum used. Nevertheless it was found that this bacterium produced large quantities of penicillinase. Thus it seems as if the varying ability of the microbes to produce the penicillin-destroying enzyme does not solve the problem of the importance of the inoculum for determination of penicillin resistance. This finding can possibly be compared with the fact that a microbe may be very sensitive to penicillin and yet produce penicillinase in abundance. This applies, for instance, to the *Bacillus subtilis*. The varying degree of resistance to penicillin shown by the bacteria is thus not solely dependent on their having the ability to produce penicillinase.

We also carried out a number of experiments in order to investigate the power of the various bacteria to form penicillinase. It was of especial interest to obtain filtrates containing the penicillin inactivator. We worked chiefly with a single strain of the *Proteus morgani*. Bouillon-cultures 7 seven days old were centrifuged and then passed through a Chamberland filter. Titration of the inactivator in the filtered bouillon-culture was carried out in the following manner: Two series of tubes, each containing $\frac{1}{2}$ ml of bouillon, were employed, and the penicillin was diluted in the same way as for titration of the penicillin resistance. To each tube in the first series was added $\frac{1}{2}$ ml of filtrate

and to each tube in the second series $\frac{1}{2}$ ml or ordinary bouillon. The latter series served for control. To all the tubes in both series was then added a staphylococcal culture in a dilution of 1/100000, likewise here $\frac{1}{2}$ ml to each tube. The staphylococcal strain employed was highly sensitive to penicillin. Thereupon followed incubation for 20 hours at 37° C. In one of the tests with the above-mentioned strain of *Proteus morgani* it was found that $\frac{1}{2}$ ml of filtrate inactivated 100 units of penicillin. Meanwhile, the results varied greatly. The variations were, in our opinion, so great that they could hardly be attributed to irregularities in technique, since the experimental conditions were all the time the same.

A more exact examination of the production of penicillinase in our *Proteus morgani* strain was therefore made. For this purpose were prepared agar-plates containing penicillin in different concentrations as well as a penicillin-sensitive staphylococcus strain. Both the penicillin and the staphylococci were added to the fluid agar at 45° C, whereupon the agar was at once poured out on dishes and cooled down to 4° C. To 100 ml of agar there was added 1 ml of a 20-hour culture of staphylococci in ordinary bouillon, and the penicillin concentration was just sufficiently strong to prevent growth of the staphylococci at 37° C.

The Morgan strain were now spread out on these plates and then incubated. Originally it was intended to investigate whether the separate colonies showed equal power of producing penicillinase. It was, however, not found possible in this manner to ascertain whether such was this case. The individual colonies of *Proteus morgani* did not produce sufficient penicillinase to neutralize the effect of the penicillin in their immediate vicinity. We therefore proceeded to inoculate in depots in such manner that one colony from each original dish formed a depot. As a rule four colonies were inoculated on each agar-plate containing penicillin and staphylococci, so that after incubation there came growth from four depots. Often after 24 hours' incubation, but regularly after 48 hours, there could now be observed growth of staphylococcus colonies beneath and around the depots of *Proteus morgani*, as an indication that the effect of the penicillin had here been neutralized or weakened. After a long series of experiments with our *Proteus morgani* strain we succeeded in proving that the penicillin-inactivating substance was produced in unequal quantities from the different depots. In some individual cases this was strikingly evident, seeing that on the same plate there might come abundant growth of staphylococci under and around a depot of *Proteus morgani*, while one or more other depots were incapable of neutralizing the effect of the penicillin, and here there came no growth of staphylococci. The conclusion to be drawn from these experiments would then seem to be that the individual cells or the individual groups of cells produced the penicillin-inactivator in varying quantities. This should then at

any rate partly explain the irregularities that arose during our attempts to produce penicillinase by filtration of bouillon cultures. We are quite aware that the technique adopted in these experiments is rather inexact. It is especially important in such investigations that the individual depots shall be of the same size and that they shall grow up with the same rapidity. We believe we have paid sufficient regard to these points. The power of producing penicillinase varied greatly. Thus on inoculation from penicillinase-negative depots there came in most cases growth of penicillinase-producing depots. The reverse situation might also be observed. The most natural explanation would be that there were split off dissociation forms, some with and some without the power of forming the penicillin-inactivating enzyme. The methods of investigation, however, were, as we have said, not very exact and we cannot disregard the possibility that a certain number of cells, for example, in a penicillinase-negative depot, may have been capable of forming penicillinase, even if this fact could not be noted by means of our penicillin staphylococcus agar plates.

Summary.

1. Penicillin sensitivity was investigated in 10 strains of *Proteus vulgaris*, 10 strains of *Proteus morgani*, 23 strains of *Escherichia coli* and 10 strains of coliform bacteria. *Proteus vulgaris* was relatively sensitive, while *Proteus morgani* was highly resistant to penicillin. *Escherichia coli* and the coliform microbes occupied an intermediate position.
2. On titration of the penicillin sensitivity in some strains it was found that the amount of the inoculum was of great importance as regards *Proteus morgani*, whereas it was without significance for the titration of *Proteus vulgaris* and *Escherichia coli*.
3. It was natural to associate the importance of the inoculum for the titration with the varying ability of the microbes to produce penicillinase, seeing that *Proteus morgani* formed large quantities of penicillinase, whereas the *Proteus vulgaris* did not produce that enzyme. Titration of the *Escherichia coli* showed, however, that also for this microbe, which produced penicillinase in abundance, the amount of inoculum used was without any influence on the result of the titration.
4. It was found that *Proteus morgani* split off cells or groups of cells with unequal power of producing penicillinase. Some cells produced penicillinase in abundance, while others seemed to produce none or else only small quantities. Meanwhile, the methods of investigation employed were not very exact, and this fact reduces in some degree the values of the experiments.

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OBSERVATIONS ON EXPERIMENTAL DENTAL CARIES THE EFFECT OF PURIFIED RATIONS WITH, AND WITHOUT DIETARY FAT

By *Humberto Granados, Johannes Glavind and Henrik Dam.*

(Received for publication November 14th, 1947).

Among the animals previously used for the study of experimental caries, the monkey has been shown to be particularly well-suited (Granados, 1944; Shaw, Elvehjem and Phillips, 1945). However, its high cost limits very much its use at the present time. On the other hand, the Syrian hamster, a less expensive animal, has been found to possess definite advantages over the rat for the study of experimental caries (Arnold, 1942).

In the literature there appear some observations related to the effect of dietary fat upon the incidence of caries. In humans, Boyd (1926) and Boyd and Drain (1928) have observed a definite arrest of caries progress in diabetic children after being submitted for some time to a diabetes-controlling diet, which differs from the usual adequate diet for a normal child in that fat, instead of carbohydrate is used as the main source of energy. All children had been maintained on the same ratio of protein-carbohydrate-fat: 7:9:21. Mellanby (1934), studying in children the effect of supplements of vitamin D upon the incidence of dental caries, observed that olive oil, a fat which scarcely contains any known vitamin, when given instead of cod liver oil, also offered a partial protection against tooth decay. Rosebury and Karshan (1939 a), on the other hand, found no correlation between the high fat content of the primitive Eskimo diet and the freedom from caries which these people exhibit. In rats, the latter workers (1935, 1939 b), studying the influence of vitamin D and fats on caries incidence, observed that corn oil produced the same significant reduction in carious lesions when it was incorporated in the diet alone, or when it was fed as a vitamin D-corn oil. Olive oil, cotton seed oil,

and lard were as effective as corn oil in decreasing the caries incidence. Schweigert, Shaw, Zepplin and Elvehjem (1946), and Schweigert, Potts, Shaw, Zepplin and Phillips (1946), studying the effect of protein, fat and carbohydrate on the incidence of carious lesions in the Cotton Rat, have observed a progressive reduction of caries incidence and extent when increasing amounts of the dietary sugar were replaced by lard. Since in the hamster there has been no observations on this subject, that can be taken into consideration for further experiments, and having chosen this animal to carry out certain studies on experimental caries¹⁾, we report here the effect of purified diets with, and without dietary fat.

Experimental.

Twenty-eight hamsters between 22 and 25 days of age, from 5 litters of the stock colony maintained on a diet of Purina Laboratory Chow²⁾ and raw milk, were litter mate distributed into 2 groups of 14 animals each (7 males and 7 females). The animals were marked, set in screen bottom cages without bedding, and reared for 105 days on the following rations. Group 1 (fat-free diet): casein (gasoline-extracted) 221 g; confectionery sugar 730 g; salt mixture³⁾ 40 g; choline chloride 4 g. The vitamins were supplied, for each Kilo of diet, as follows: p-aminobenzoic acid 1 g; inositol 1 g; thiamin hydrochloride 0.050 g; riboflavin 0.050 g; pyridoxine 0.050 g; calcium pantothenate 0.050 g; nicotinic acid 0.075 g; folic acid 0.004 g; biotin 0.0002 g; vitamin K (Synkayvite) 0.030 g; vitamin E (,1- α -tocopherol acetate) 0.050 g; and ascorbic acid 0.200 g. Group 2 (fat-containing diet): 93 % of the diet fed to Group 1 mixed with 7 % lard. Each animal of both groups received 2 drops weekly of a solution of vitamins A and D in oleic acid, containing 22,000 I. U. of A, and 320 I. U. of D per g. The diets as well as tap water were available ad libitum, and the animals were weighed every week. These experiments were carried out between the months of April and August.

On completion of the experimental period the animals were sacrificed and autopsy was performed on all of them. After fixation in 10 % formalin the jaws were washed, stripped of soft tissue and dried. The teeth were then examined under a low-power dissecting microscope ($\times 15$), and the gross, unquestionable, carious lesions were charted and scored using Keyes' method (Keyes, 1944).

¹⁾ Two of us (Granados and Dam) begun such studies in 1945, while at the University of Rochester School of Medicine and Dentistry, Rochester, N. Y., U. S. A.

²⁾ From Ralston Purina Company, St. Louis, Mo., U. S. A.

³⁾ The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KI, 139 mg CuSO₄ · 5H₂O, and 556 mg MnSO₄ · 4H₂O per 100 g.

Results.

The animals from group 1 exhibited a pronounced dryness of the fur and skin, with scaly appearance of the latter. These changes were apparently associated with a deficiency of essential unsaturated fatty acids. Likewise, the fat depots were exceedingly reduced, as compared with those of the animals from group 2. Figure 1 shows the growth curves of the two groups. Group 1 (fat-free diet) exhibits a marked decrease of growth rate.

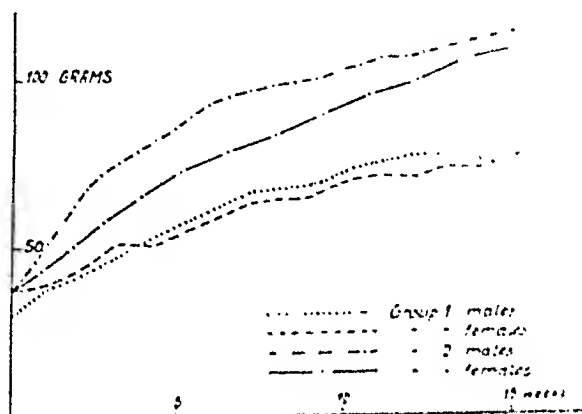


Fig. 1.

Table 1.
Caries incidence in the two groups.

Number of Experimental Animals ...	Group 1			Group 2		
	7♂♂	7♀♀	7♂♂ + 7♀♀	7♂♂	7♀♀	7♂♂ + 7♀♀
Number of Animals Affected	7	7	14	7	6	13
Number of Carious Molars	36	31	70	29	23	52
Average Number of Carious Molars ..	5.14	4.80	5.00	4.14	3.28	3.70
Number of Carious Lesions	38	39	77	33	23	56
Average Number of Carious Lesions ..	5.42	5.57	5.50	4.71	3.28	4.00
Average Scores of Carious Lesions ..	0.591	0.961	0.778	0.758	0.492	0.626

Upon observation of the fresh appearance of the oral mucosa no gross changes were encountered in either group. Materia alba-like accumulations, not associated with caries, were observed on most molars in all the animals of both groups. Likewise, a yellow-brown stain of the enamel cuticle, unrelated to caries activity, was found on all surfaces of most molars in both experimental groups. This stain seemed similar to that found by Keyes (1946).

Both diets induced a very low incidence of caries. All the lesions, with a few exceptions in some grooves, were encountered in the occlusal fossae. The smooth surfaces were consistently unaffected. The lesions were of a penetrating type, reaching in many instances the pulp, with only little of surface destruction. Comparing the caries incidence in the two groups (Table 1), lower figures are found in group 2. Comparing the caries activity in the two jaws (Table 2), in both groups the greater incidence and extent of lesions are found in the

Table 2.
Caries incidence in individual molars.

	Molar teeth	Group 1			Group 2		
		♂♂	♀♀	♂♂+♀♀	♂♂	♀♀	♂♂+♀♀
Percentage of Molars affected	Maxillary						
	1st	42.8	50.0	46.4	21.4	7.1	14.3
	2nd	78.6	64.3	71.4	85.7	50.0	67.8
	3rd	64.3	71.4	67.8	78.6	78.6	78.6
	Mandib.						
	1st	0	0	0	0	0	0
	2nd	28.6	7.1	17.1	7.1	0	3.6
	3rd	42.8	50.0	46.4	14.3	28.6	21.4
	Maxillary						
Average Percentage of Caries Lesions	1st	57.1	50.0	53.6	21.4	7.1	14.3
	2nd	78.6	64.3	71.4	100.0	50.0	75.0
	3rd	64.3	92.9	78.6	92.9	78.6	85.7
	Mandib.						
	1st	0	0	0	0	0	0
	2nd	28.6	7.1	17.9	7.1	0	3.6
	3rd	42.8	64.3	53.6	14.3	28.6	21.4
	Maxillary						
	1st	0.595	0.661	0.628	0.265	0.066	0.165
Average Scores of Carious Lesions	2nd	0.967	1.116	1.042	1.711	0.814	1.265
	3rd	1.181	3.740	2.461	2.760	2.165	2.461
	Mandib.						
	1st	0	0	0	0	0	0
	2nd	0.298	0.074	0.186	0.074	0	0.037
	3rd	0.670	0.893	0.781	0.298	0.372	0.335

maxilla. This agrees with previous findings by Dale, Lazansky and Keyes (1944), and Orland (1946 a). Comparing the caries activity as found in individual molars, Table 2 shows, further, that the greatest incidence and extent of lesions were found in the 3rd molars of both jaws, the 1st molars being the least involved. A striking feature was the complete absence of caries in the 1st mandibular molars of all the animals in both groups. Tables 1 and 2 also show that no sex difference was consistently found in relation to caries activity.

Discussion.

Up to the present, in the reported studies on dental caries in hamsters, the cariogenic diets used, with one exception, have been mixtures of natural food-stuffs. On the other hand, the use of purified cariogenic diets has the advantage that it makes it possible to ascertain more clearly the influence of certain components of a diet. In our previous studies on dental caries in hamsters, while at the University of Rochester (1945), we observed high incidence of caries induced by purified rations⁴⁾. However, in this country similar diets, such as those used in the present study, have proved of a low cariogenic power. Certain physical or chemical differences should account for these variations: previous treatments of the caseins and difference in the size of their granules; components of the salt and vitamin mixtures, and kinds of sugars and fats⁵⁾.

Orland (1946 b), working with purified rations containing 10 % hydrogenated vegetable oil, also obtained caries scores appreciably higher than those shown here (Table 1) for any of the two groups. His diets I and VIII are comparable, in general, with the fat-containing ration of this report. However, certain differences are apparent: previous treatments of the caseins, kinds of fats, components of the salt mixtures, and presence of ascorbic and folic acids in the diets used in the present studies. It should prove important to know the mechanism whereby such, or other less apparent, differences made the diets used in the present experiments less cariogenic. It should be mentioned that in the diets used by Orland the source of fat was vegetable oil submitted to hydrogenation, a process which annuls the unsaturated fatty acids. It is well known that oleic and linoleic acids are constantly

⁴⁾ The composition of the diet previously used was as follows: SMACO vitamin-test Casein 309 g; dextrose (chemically pure) 600 g; salt mixture (U. S. P. XII) 40 g; choline chloride 0,50 g; Corn oil 50 g. The vitamins were supplied, for each Kilo of diet, as follows: thiamin hydrochloride 50 mg; riboflavin 50 mg; calcium pantothenate 50 mg; pyridoxine 50 mg; nicotinic acid 75 mg; sodium p-aminobenzoate 100 mg; Inositol 100 mg; biotin 0,4 mg; vitamin K (Synkayvite) 10 mg. Two drops of Haliver oil were given weekly to each animal. Tap water was available ad libitum.

⁵⁾ The possibility of a protective action of the water due to its high fluorine content can be excluded since the tap water of Copenhagen has a fluorine content of only 0,4 to 0,8 parts per million.

found in lard, as well as in most fats; these unsaturated fatty acids have been shown to exert, in some instances, definite inhibitory action on the growth and lactic acid production of certain microorganisms, some of them commonly found in the mouth (Kodicek and Worden, 1944, 1945; Bergström, Theorell and Davide, 1946; Hirsch, 1947). On the other hand, other investigators (Dubos, 1946; Hutchings and Boggiano, 1947; Williams, Broquist and Snell, 1947) have reported that oleic acid and related compounds are growth factors for certain bacteria also generally found in the oral cavity. Thus, as to the significance of oleic and linoleic acids in relation to any inhibitory action of fats in dental caries, nothing can be said with certainty at the present time.

The partial protection against caries induced by the fat-containing diet, under the conditions of our experiment, was of a low statistical significance.

The fact that Group 2 exhibited a better growth (Fig. 1), concomitant with lower caries activity (Table 1), could point to an explanation of the protective role of lard in dental caries through its general nutritional value as a fat. However, the clinical and experimental observations on this subject are very contradictory. Pointing to a local effect, McCollum, Orent-Keiles and Day (1939), and Rosebury and Karshan (1936 b) have suggested that free fat may exert a lubricating action, preventing food impaction, or may coat both food particles and adjacent tooth surfaces with a non-aqueous film, which forms a hindrance to the penetration of enzymes into the particles, or affording protection of the enamel against the acids derived from carbohydrate fermentation.

Summary.

Two groups of young hamsters were reared for 105 days on purified diets with, and without lard. In agreement with the reports of other investigators working with rats, the hamsters receiving the fat-containing ration showed a lower incidence and extent of carious lesions, though the differences were of low statistical significance. The group fed the lard-containing diet exhibited a healthier appearance and a higher growth rate than that reared on the fat-free ration.

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PREPARATION OF PURIFIED ANTITOXINS

By *Albert Hansen.*

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Introduction.

In continuation of previous works (1931, 1938, 1938, 1941) the writer has extended his studies on the preparation of the purified antitoxins, partly for the purpose of obtaining an increased effect of the antitoxin, partly to abolish the noxious by-effects due to superfluous protein content of the antitoxin.

The method described in the following for the preparation of purified antitoxins is based on experiences gathered by the writer in experiments on purification of various native products by means of adsorption — including purification of the antitoxin in diphtherial sera by their adsorption on aluminium hydroxide gel. The experiment showed that aluminium hydroxide gel adsorbs the serum protein as a whole, and that the adsorption thus does not involve any shifts in the ratio of antitoxin/protein.

A few diphtherial sera in which there had been bacterial growth — among others, of *Bac. pyocyaneus* — deviated in the way of their adsorption, the unspecific protein being adsorbed in preference to the antitoxin-carrying protein. Thus the adsorption implied a certain purification of the antitoxin. Subsequent experiments were carried out with exposure of diphtherial sera to the action of bacterial proteinases and simultaneous proteolysis with pepsin, trypsin and papain prior to the adsorption.

The various enzymes had a more or less pronounced effect on the proteins in the diphtherial serum similar to that of the bacterial growth. The selective adsorption of unspecific proteins was obtained, implying thus a purification of the diphtherial antitoxin.

Adsorption of dilute diphtherial serum that had been exposed to the action of pepsin in acid fluid proved to give more promising results than adsorption after exposure to other proteolytic enzymes.

So in the following studies pepsin was used exclusively for proteolysis of the serum prior to the adsorption. At a temperature of 20° the antitoxins in dilute sera are relatively stable in an acid milieu. Thus the antitoxins are able to stand an acidity of $\text{pH} = 3.20$ as the lower limit. Even at $\text{pH} = 3.10$ the antitoxin weakens rapidly and inconsiderably.

When dilute diphtherial serum is acidified to $\text{pH} 3.20$ and then a suitable amount of pepsin is added, the following proteolysis manifests itself noticeably in a gradually progressive shortening of the flocculation time — when the diphtherial antitoxin is assayed in vitro after Ramon's method — until, as a rule after 12 hours, it reaches a constant minimal value. (At the same time there is a shift between the flocculation titer and the titer obtained in vivo, the former being reduced by 10—20 %). Shortening of the flocculation time is accompanied by liberation of the antitoxin-carrying protein from the unspecific concomitant proteins, which makes it possible to remove the latter by adsorption. When the flocculation time for the diphtherial antitoxin has reached its constant low value, the conditions for removal of the unspecific serum protein are the best possible in the individual cases.

In the following a survey will be given of how the writer employs the above-mentioned observations for practical purification of antitoxins.

Removal of Antiproteolytic Serum Factors.

The proteolytic action of pepsin in antitoxic sera is inhibited in a higher or lesser degree by certain substances present in the serum — as was mentioned frequently in the older literature. Occasionally this inhibition may be so marked that quantities of pepsin generally giving pronounced proteolysis now become practically without any effect. In such cases, therefore, the amount of pepsin added had to be increased in order to obtain proteolysis, and this increased addition of pepsin led to destruction of a smaller or greater part of the antitoxin.

So the antiproteolytic factors had to be removed, and this was attempted in various ways: by filtration through activated charcoal, by fractionated salting, by extraction with various solvents, and by precipitation with alcohol at a low temperature. Several of these treatments had a somewhat favorable effect, but far better results are obtained by addition of a suitable amount of bentonite to dilute serum. The strong precipitate of coagulation products then contains, besides remnants of fibrin and euglobulin, also cholesterol and lipoids. After centrifuging of the precipitate, the supernatant fluid is clear as water. At any rate, it is rid of antiproteolytic factors.

Bentonite (montmorillonite) is obtained commercially as a

greyish-yellow powder with a particle size of about 200 micro μ . On stirring with distilled water, bentonite forms a stable sol which on addition of electrolyte changes into a gel with pronounced thixotropic properties. If an excess of electrolyte is added to the sol, an irreversible coagulum is formed. On addition of a solution of fibrinogen or euglobulin to bentonite sol, a mutual coagulation results. The coagulation product precipitates spontaneously. If, conversely, a suitable amount of bentonite sol is added to freshly withdrawn blood, the fibrinogen content of the blood coagulates together with a large part of the euglobulin. In their precipitating, the clotting products adsorb a greater part of the lipid content of the blood.

In the course of the present work there has been occasion to compare the effect of a number of different bentonite samples, and the most suitable for this particular purpose was found to be a commercial produkt from U. S. A. This substance, which hitherto has been used exclusively in the preparation of antitoxins is composed as follows:

Si O ₂	: 49.56 %
Ti O ₂	: 0.40 %
Al ₂ O ₃	: 15.10 %
Fe ₂ O ₃	: 3.03 %
MnO	: 0.01 %
CaO	: 1.10 %
MgO	: 7.80 %
H ₂ O	: 23.00 %

Invariably an aqueous sol is employed that contains 2 % bentonite. The sol may be prepared by stirring the bentonite in a dish under gradual addition of water. But it is better to employ a stirring machine, and then the distilled water is first poured into the dish whereafter — under stirring — the powder is shaken out on the surface of the water. The stirring is continued for 2 hours. The sol is autoclaved for 30 min. at 121° (the autoclaving costs about 30 % increase in the activity). After cooling and replacement (by weight) of the water evaporated on autoclaving, the sol is ready for use. On standing, a sediment is formed that has to be stirred up before using. The sol keeps indefinitely when it is stored in bottles of resistant glass.

The amounts of bentonite sol required for precipitation of the antiproteolytic factors of a serum vary from one serum to another. As an excess of sol beyond the precipitation limit brings about a loss in antitoxin and thus should be avoided, it is necessary to titrate the individual serum. For this titration, the dilute serum is distributed on a series of test tubes, to which bentonite sol is added in increasing amounts. In one of the tubes a marked flocculation will appear within some minutes, and from the amount of bentonite sol in this tube

then the amount required for precipitation of the main portion is calculated. When the precipitation with bentonite sol is performed in the proper proportion, it gives practically no loss of antitoxin.

A general survey of the procedure adopted for the preparation of purified antitoxins is given in Fig. 1.

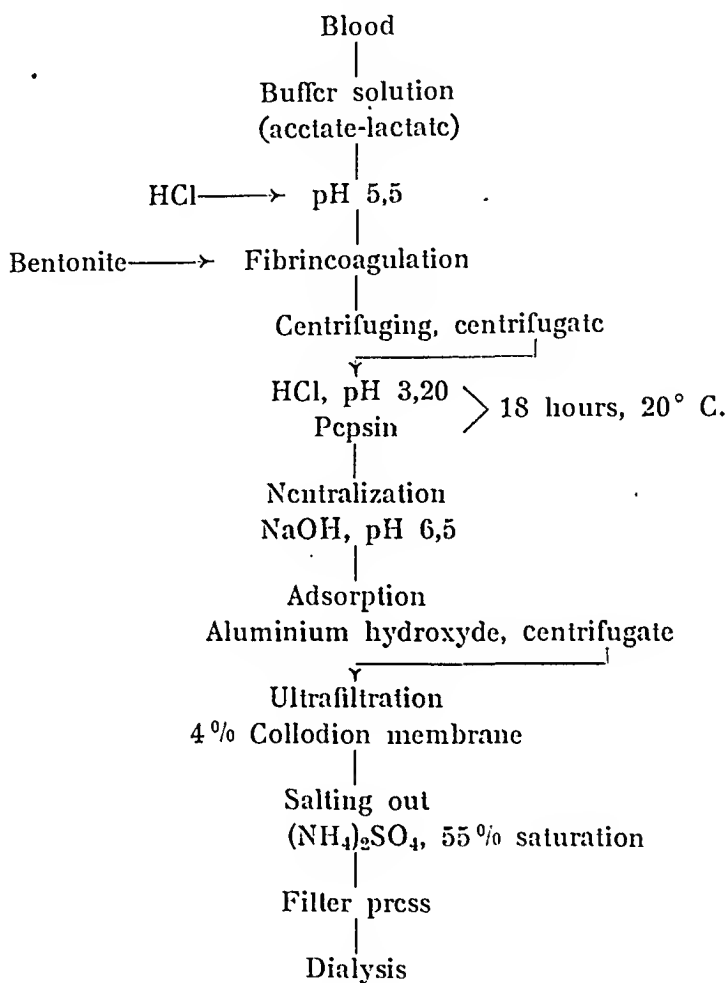


Fig. 1.
Diagrammatic survey of the procedure for preparation of purified antitoxin.

Materials, Apparatus and General Procedure.

1. Bleeding.

In the preparation of purified antitoxin it is of the greatest importance to the final yield that the antitoxin-carrying protein is obtained in the greatest possible yield and in a form unaffected by the bleeding. These requirements are not met satisfactorily by the me-

thod commonly employed for the preparation of the serum. After this method, as is well known the withdrawn blood is left standing for clotting whereafter the serum is separated by synaeresis of the clot. (By extraction of the pressed clot (waste product in the preparation of antidiptherial serum) the writer was able to recover an amount of antitoxin of 10—15 % of the serum corresponding to the clot.)

In contrast hereto, the bleeding gives a quantitative yield of antitoxin, when the blood is withdrawn under employment of bentonite sol as fibrinogen-precipitating adjuvant.

On addition of bentonite sol to freshly withdrawn blood the fibrinogen coagulates spontaneously as tiny particules. Thus the clotting of the blood is prevented at the same time as the blood serum is rid of its antiproteolytic factors.

The coagulation products together with the blood corpuscles may be removed from the dilute serum by centrifuging immediately after the addition of bentonite sol to the diluted blood. The centrifugate forms a good starting material for the preparation of purified antitoxins. Usually 8 liters of blood is withdrawn from each horse.

Before the bleeding the following reagents are made up for each 8 liter:

1. *Buffer solution*: 130 cc. 80 % lactic acid
 155 g. sodium acetate
 52 « sodium hydroxide
 125 « sodium chloride.

The salts are dissolved in distilled water, and the volume is made up to 22 liters. Then the acidity is adjusted to pH 5.5.

The solution is poured into 40-liter aluminium containers that were sterilized by autoclaving and cooling.

2. *Hydrochloric acid*: 450 cc. n/1 HCl diluted to 500 cc. with distilled water.

3. *Bentonite sol*: 1280 cc. 2 % bentonite sol.

When the blood is to be withdrawn, a bucket containing the buffer solution is placed on a scale in a room adjoining the bleeding room. From the cervical vein of the horse the blood is quickly led through a tube placed in the wall to the bucket containing the buffer solution until the contents of the bucket weigh 8480 g., corresponding to 8 l. blood. As a rule, it takes 4 minutes to withdraw this amount of blood.

Then, under cautious stirring the hydrochloric acid is added, bringing the acidity of the mixture up to pH 5.5, and then the bentonite sol is added at once. The entire process from the commencement of the bleeding to the end of the bentonite sol addition takes about 6 min.

At the bleeding the blood is collected from a group of horses with nearly the same antitoxin content. Thus the horses immunized against diphtheria are divided into groups with a serum titer of 300 A. U. per cc., groups with a serum titer of 500 A. U. per cc., other groups with the titer of 800 A. U., and so on.

The contents of the bleeding buckets from such a group of horses are mixed in a container from which it is led to a separator centrifuge (manufactured by »Titan«, 6000 revolutions per min.) which separates the serum protein solution from the heavier fluid made up of the bentonite fibrin coagulation product and blood corpuscles.

The serum protein solution is still a little opaque. Its final clearing is obtained by repeated precipitation with bentonite sol.

Portions of 10 cc. centrifugate are transferred by pipette to a series of 12 test tubes, to which distilled water then is added in amounts of from 11 to 0 cc., and finally dilute bentonite sol (1 + 9) in distilled water is added in amounts increasing from 1 cc. to 12 cc. Thus the total amount in each tube will be 22 cc. After cautious mixing and standing for some minutes, one of the tubes will show a marked flocculation, in which sedimentation of the floccules leaves a clear supernatant fluid.

Then the main portion is precipitated with the calculated amount of bentonite sol. (If, for instance, the flocculation in the tube 4 was found to correspond to 4 % bentonite sol, and if the main portion amounts, for instance, to 500 liters, the precipitation is performed with 20 l. bentonite sol.) For addition to the main portion, the bentonite sol is finely atomized on the surface of the fluid while this is being stirred vigorously.

After standing for 10 min., the coagulation product is removed from the fluid by centrifuging (with employment of the same »Titan« separator as above).

2. Proteolysis.

The clear centrifugate from the second bentonite precipitation is led up into a container of stainless steel, in which the fluid is acidified to pH 3.20, the required amount of $n/2$ HCl being added under vigorous mechanic stirring.

The hydrogen ion concentration is measured with glass electrode. The comparative fluid is a solution consisting of

glyecoll	1 g.,
glacial acetic acid 99 %	2 cc.,
distilled water q. s. ad	100 cc.

This buffer solution has pH 3.28.

Simultaneously with the acid, the calculated amount of pepsin solution is added. The latter is calculated so that the pepsin content

of the proteolytic fluid corresponds to 2 U. S. P. units of pepsin per cc. (If, for instance, a standard pepsin solution is employed with 1:600 U. S. P. per cc., and if the volume of the given proteolytic fluid is 600 liters, 2000 cc. pepsin standard solution will have to be employed.)

The proteolysis takes place at 20°. Still, deviations of $\pm 1^\circ$ are safe; and as in our manufacturing rooms the temperature most often is 20°, it happens but seldom that the incubator has to be employed for this purpose.

In the first 60 min. of the proteolyzing period a minor shift of pH takes place in the proteolytic fluid. Therefore a pH = 3.20 is again established by addition of hydrochloric acid. From now on the acidity of the fluid keeps unchanged, and any further pH control in the course of the proteolysis is superfluous. After 18 hours the proteolysis is interrupted by adding, under vigorous mechanical stirring $n/2$ NaOH sufficient to give the proteolytic mixture an acidity of pH = 6.5. (This neutralization has to be carried out very cautiously, as antitoxins are very sensitive to alkali.)

Pepsin.

In the first years the proteolysis in antitoxin preparation was carried out most often with pepsin from Parke Davis & Co., which gave very satisfactory results. When the world war broke out in 1939 we had to employ pepsin from various other sources, which was associated with a good deal of trouble. For one thing, the activity of the pepsin was rarely in conformity with the declaration, and sometimes the received preparations possessed no activity whatever, or they contained large amounts of mucin that had to be removed before using the pepsin. Besides, sometimes the pepsin might contain impurities that would have a destructive effect on the antitoxin. In some cases, for instance, the pepsin preparations were demonstrated to contain heavy metal salts, and in one instance the pepsin was found to contain an impurity consisting of a protein with pronounced anaphylactogenic properties.

These experiences illustrate the necessity of caution in employment of commercial pepsin for this particular purpose. Thus we found that in the preparation of purified antitoxin it would be safest ourselves to prepare the pepsin required for this purpose, and, by doing this, in recent years we have had no difficulties of this character. At short intervals we still prepare a relatively small portion of pepsin solution from fresh swine stomachs. Thus the pepsin here employed is never more than a few months old.

Example of preparation of pepsin:

250 kg. fresh swine stomach are freed from mucus by scraping without washing, the fundus region of each stomach is cut out and the mucous membrane here is pulled off. This gives a total yield of

about 30 kg. fundus mucosa, which is placed in a cylindric jar of earthenware, with a capacity of 50 liters. The content of the jar is worked by a strong mechanical stirring contrivance. The following solution is worked into the mass:

Crystalline secondary sodium phosphate	500 g.
Concentrated hydrochloric acid	600 cc.
Water ad	10 l.

This mass is left standing at room temperature under continuous mechanical stirring with control examination of its acidity at suitable intervals, which gradually is brought down to pH ca. 2 by addition of hydrochloric acid and shown in the following example:

29/5, at 13,45: Working up of fundus membranes as described.
 « 14,45: pH of mixture 3.15. Addition of 100 cc. conc. HCl.
 « 16,15: « « « 2.90. « « 100 « « «
 « 21,00: « « « 3.00. « « 100 « « «
 30/5, « 9,30: « « « 3.15. « « 100 « « «
 « 10,30: « « « 2.65. « « 100 « « «
 « 14,00: « « « 2.70. « « 100 « « «
 « 16,00: « « « 2.40. « « 50 « « «
 « 17,30: « « « 2.35. « « 50 « « «
 « 24,00: « « « 2.40. « « 100 « « «
 31/5, « 8,30: « « « 2.25. « « 50 « « «
 « 9,00: « « « 2.00. The jar is now transferred to
 a water-bath at 37°, where it is left standing without stirring.
 31/5, at 16,00: pH of mixture 1.95. (The mixture is now clear).
 1/6, « 10,00: « « « 2.20. Addition of 75 cc. conc. HCl.
 « 21,00: « « « 2.00.
 2/6, « 9,00: « « « 2.05.
 « 16,00: « « « 2.05. The mixture is cooled to 10°, and
 its acidity lowered by addition of 400 cc. concentrated ammonia. Now
 the mixture has pH = 3.20. The 45 kg. hydrolysate is evaporated in
 vacuum at 26° to 21 kg.

The evaporation rest is placed in cellophane tubes and dialyzed against 300 l. tap water acidified with HCl to pH 3.20. The dialysis is continued for 48 hours, during which the dialysis water is changed 5 times. The dialysate, 37 kg., is evaporated in vacuum to 14 kg. The evaporation rest is chilled to 0° and then, under vigorous stirring mixed with 9250 cc. acetone of ÷5°. The precipitated mixture is centrifuged. The clear centrifugate, 14500 cc. is freed from acetone by evaporation in vacuum to 8000 cc. The strongly turbid evaporation rest is clarified by precipitation with bentonite sol after the precipitation limit has been established (which is performed by employment of the same technique as described under »Bleeding«, where the serum protein solution was freed from its content of antiproteolytic factors by

precipitation with bentonite sol). For this titration the evaporation rest is diluted 1 + 9 with distilled water, and the bentonite sol is diluted with 1 + 9 distilled water. In the above-mentioned preparation the flocculation in tube 4 was found to correspond to 40 % bentonite sol.

So 3200 cc. bentonite sol was added to the evaporation rest, 8000 cc. The precipitated mixture was centrifuged. By cautious addition of ammonia the clear centrifugate was adjusted to pH 3.50 and finally mixed with an equal volume (9800 cc.) of chemically pure glycerin. The finished preparation was sterile-filtered through Seitz E. K. plates.

During the preparation the pepsin activity was controlled continuously.

In the preparation outlined above the following values were found for the pepsin activity:

In autolysate after add. of ammonia,	1:810	per cc.;	total ca.	30.8	millions	
« dialysate	1:810	«	«	«	29.2	«
« evaporation rest aft. acetone add.	1:2600	«	«	«	20.8	«
« centrifugate after bentonite add.	1:1620	«	«	«	15.9	«
« finished product after filtration	1:800	«	«	«	15.7	«

(The sample taken before addition of glycerin and evaporated to dryness after freezing showed an activity of 1:18000 per g. dry matter.)

The finished preparation is stored in refrigerator at 0°. Its activity is found to keep without any demonstrable weakening after 6 months' storage.

The *pepsin activity* after a method in which the acetone titration of amino acids given by Linderstrøm-Lang is employed. As standard in the elaboration of the titration curve we have used a pepsin from Parke Davis & Co. and another pepsin from »Difco« with a guaranteed strength of 1:3000 and 1:10000, respectively.

The following solution are made up:

1. *Casein Substrate*. — A casein solution is prepared in this way: 6 g. casein, Hammersten is placed in a 100-cc. volumetric flask, and 30 cc. distilled water is added; the flask is shaken thoroughly so that the casein becomes moist throughout. Then 36 cc. n/10 NaOH is added in small portions. After addition of 1 drop octylic alcohol and 2 drops toluene, the flask is left standing at room temperature, under frequent shaking. When all the casein is dissolved the flask is filled to the mark with distilled water.

The solution is placed in refrigerator at + 2° and can be stored there for 1—2 months.

The casein substrate is prepared in a glass as shown in Fig. 2. Into one branch of this glass, 25 cc. of the casein solution is transferred by pipette, while 4 cc. 0.63 n HCl is placed in the other branch. This

amount of acid will bring the casein solution to pH 2.15. For the mixing of these components, by a quick movement the casein solution is thrown over into the hydrochloric acid, and then the glass is shaken energetically for 60 sec.

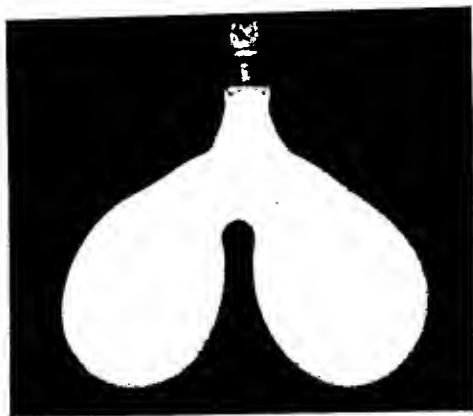


Fig. 2.
Glass for preparation of casein substrate.

2. *Pepsin Dilution.* — The pepsin dilutions are made up with distilled water that is acidified by adding 1 drop 80 % lactic acid to 2 liters distilled water. The degree of dilution is chosen so that 1 cc. dilution will correspond from 0.3 to 1.2 pepsin units.

3. *Acetone with Dissolved Indicator.* — For indicator under the titration we employ naphthyl red (benzolazo- α -naphthylamine) of this dye, a solution is made of 0.1 g. in 100 cc. 96 % alcohol. Of this dye solution, 25 drops is added to 100 cc. acetone.

For the analysis, calibrated test tubes of 12 cc. are employed — preferably tubes with a ground glass stopper (but also tight fitting corks may be employed). — 1 cc. of the pepsin solution is placed at the bottom of each tube.

At the same time, 1 cc. casein substrate is placed in a parallel series of tubes of 5 cc., paraffined inside.

For each determination of the pepsin activity 5 sets of the two tubes are made ready; and 3 of these sets are placed in a water-bath at 40°. After preliminary heating for 5 min. the first set of tubes are started by pouring the substrate from the small paraffined tube into the tube containing the pepsin dilution. After strong shaking this tube is stoppered and put back into the water-bath at 40°. At intervals of exactly 60 sec. 3 sets of tubes are started in this way. The remaining two sets are used for controls in the titration. They are mixed, and 6 cc. acetone is added to each large tube immediately after the mixing.

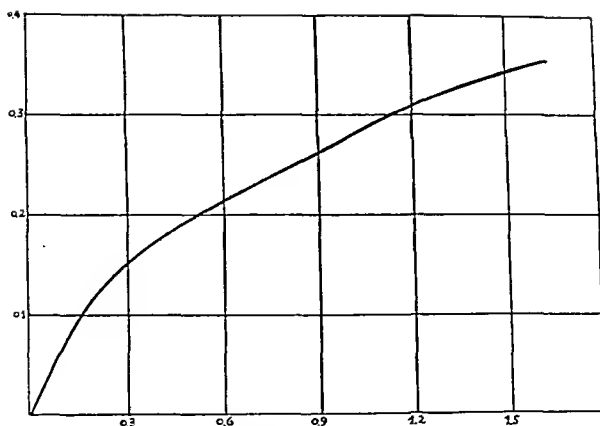


Fig. 3.

Titration curve for Pepsinactivity.

Ordinate: n/40 HCl.

Abscisse: U. S. P. Pepsin Units.

Exactly 60 min. after the mixture, the reaction tubes are removed from the water-bath, and 6 cc. acetone is added to each at intervals of 60 sec. The color in the control tubes is reddish-orange, while in the reaction tubes it is entirely yellow.

For the titration a burette is employed, divided in 0.02 cc. and containing n/40 HCl in 75% C_2H_5OH . From this burette so much of the acid is added to each of the reaction tubes that they have the same color as the controls, the change in color is estimated best when it is examined against a narrow slit of daylight.

From the amount of n/40 HCl used to obtain this change in color the pepsin activity can be read directly on the titration curve, Fig. 3.

(Example of measurement: The pepsin solution, prepared as described above, was measured in dilution 1:1000. For the titration 0.25 cc. n/40 HCl was used, corresponding to 0.8 pepsin unit. Thus the pepsin activity of the solution is $0.8 \times 1000 = 1:800$.)

Adsorption of Unspecific Protein.

It is of the greatest importance to the outcome of the adsorption as well as to the most complete utilization of the adsorbent — aluminium hydroxide — that the adsorbent and adsorptive are mixed properly. The writer has found it most expedient to add the adsorbent by blowing it in atomized form over the surface of the proteolysate, while this at the same time is being stirred vigorously, so that the surface of the fluid is being renewed continuously.

Generally the adsorption is performed in this way, that the total amount of adsorbent required is added in 4 or 5 steps, and the adsorbent from the preceding step is removed before the next addition of adsorbent takes place.

Naturally the addition of adsorbent in this way requires containers of considerable dimensions in order to provide a suitably large active surface, and also to leave room for the large amounts of foam appearing in the first steps of the adsorption. As will be evident from Fig. 3 these containers are horizontal tanks of stainless steel provided with a stirrer and manhole, besides the handhole for introduction of the centrifugate as well as the atomized adsorbent. At the lowest part of the bottom of the tank there is an outlet. After the adsorbent has been added, the handhole is closed with a screw-cover, and by means of compressed air the adsorption mixture is then forced up through the centrifuge.

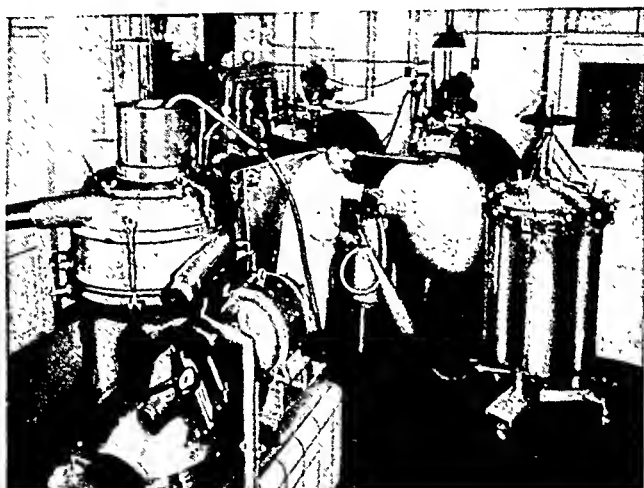


Fig. 4.
Adsorption plant (see the text).

The centrifuge here employed is a so-called »rotojector« (from »Titan« Ltd.) a 3-phase separator, which is able to separate two fluids of different specific gravity, while the firm sediment is separated and removed from the centrifuge during its revolution. The sediment is evacuated by turning a single handle: the centrifuge then opens while rolling at a rate of 6200 revolutions. After it has slung off the sediment, the centrifuge closes again automatically. The centrifugate is led from the centrifuge over into the other tank, where a new portion of adsorbent is added, and then the centrifuging is repeated. In this way the adsorption is performed twice more.

After the fourth adsorption the centrifugate becomes more clear, and a sample of it is withdrawn to see how much adsorbent has to be added at the 5' adsorption in order to remove the unspecific protein still present in the centrifugate. The sample of centrifugate is distributed on flasks, 100 cc. in each. To these portions then adsorbent

is added in increasing amounts: $\frac{1}{2}$ cc., 1 cc., $1\frac{1}{2}$ cc. and 2 cc. In the form of a fine jet (most efficiently through a needle of a hypodermic syringe), while the fluid is being shaken. In the portion where the addition of adsorbent has brought a complete adsorption of unspecific protein there will at once appear a marked flocculation of adsorbate, leaving a water-clear supernatant fluid. When poured out on a hard filter paper (baryte paper), the fluid runs through just as clearly and rapidly as distilled water — in contrast to samples where the adsorbent is added in insufficient amounts (here the still remaining protein will peptize the adsorbate, making the filtrate turbid). When thus it has been ascertained how much adsorbent is required for complete adsorption of unspecific protein in 100 cc., it is easy to calculate how much adsorbent has to be used for the main portion.

After this fifth and last adsorption the centrifugate is clear as water, even though in a relatively thick layer it often shows a slight opalescence of peptized adsorbate.

To the following ultrafiltration it is highly important that the fluid is perfectly clear (an opaque filtrate would rapidly lower the yielding capacity of the filter membrane), the adsorbed centrifugate is forced rapidly through a Seitz clearing filter.

The amount of aluminium hydroxide required for the adsorption of various proteolysates is subject to wide variation. Thus diphtherial proteolysate will generally require 20—25 % more adsorbent for the adsorption of unspecific protein than is required by corresponding amounts of tetanus or gas-gangrene proteolysate.

But also variations in the adsorptive capacity of the aluminium hydroxide gel will also manifest itself clearly in the course of the adsorption (this capacity is determined by the ability of the aluminium hydroxide gel to adsorb diphtherial toxin from a toxin broth, see below).

For the adsorption of unspecific protein from proteolysates, the writer has prepared two modifications of aluminium hydroxide gel that are about equal as to their adsorptive capacity per g. Al_2O_3 .

1. *Aluminium Hydroxide Gel made from Ammonium Alum.* — The preparation of this gel is based chiefly on the technique given by Willstatter and collaborators. Through a special process of stabilization it is obtained in a form that keeps its adsorptive capacity practically unchanged even on storage for years. Besides it stands autoclaving very well. This gel is also serviceable for the preparation of vaccine against diphtheria, tetanus, chicken pest, foot- and mouth-disease etc. It is manufactured and distributed by »Dansk Svovlsyre- & Superphosphatfabrik, Copenhagen«. This preparation contains from 1.3 to 1.4 incineration rest; and its high adsorption capacity is guaranteed.

2. Aluminium Hydroxide Gel made from Aluminium Nitrate. —

In contrast to the preceding, this preparation is very easy to make in any laboratory. It is quite serviceable and adsorbent for unspecific protein from serum proteolysates. But in contrast to the preceding aluminium hydroxide gel it is unsuitable for the preparation of vaccine.

The precipitation of the preparation takes place in the apparatus shown in Fig. 5.

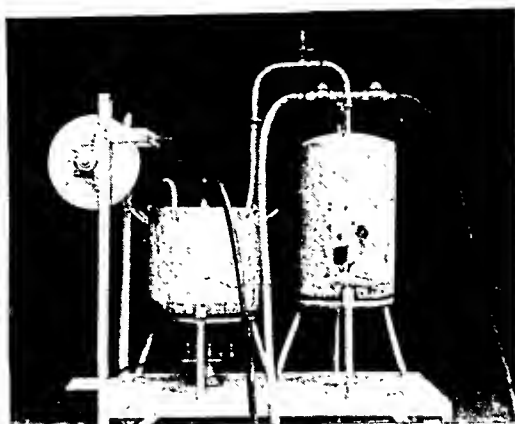


Fig. 5.

Apparatus for preparation of aluminium hydroxide gel.

The apparatus consists of the precipitation vessel and ammonia developer. The precipitation vessel is an aluminium pot measuring 36 cm. in diameter, 30 cm. in height. It is placed on a tripod and heated by a strong gas burner. This vessel is provided with a stirrer of stainless steel and a supply tube for ammonia. This tube, made of aluminium and shaped as a perforated ring, is placed on the bottom of the vessel; it is connected with the tube from the ammonia developer through a union. The precipitation vessel also contains a spiral tube of aluminium, through which cold water can be led for cooling of the reaction mixture.

The ammonia developer is a container of thick iron plate, likewise placed on a tripod. This container is provided with a tube, leading from the bottom up through the top and, externally, down below the level of the bottom of the container. Through this tube, which is provided with a valve, the reaction fluids are sucked into the container by means of vacuum, and, after using, the waste fluid is forced out through this tube by means of compressed air.

Through a tube provided with 2 side branches with valves, steam or compressed air may be led into the container. This tube goes down to the bottom of the container, where it forms a perforated ring. A thermometer may be inserted in the wall of the container.

From the top of the ammonia developer a tube leads the mixture of ammonia and air over into the precipitation vessel; and in front of the mouth of this tube, within the container, a drop catcher is placed.

For the preparation of aluminium hydroxide gel, 2500 g. aluminium nitrate — $\text{Al}(\text{NO}_3)_3$, 9 H_2O is dissolved in distilled water. The solution is made up to 20 liters and filtered on nutsche through a coarse-pored quartz filter plate. The solution is poured into the precipitation vessel and heated to 60° .

Into the ammonia developer, by means of suction, a solution of 1450 g. ammonium sulphate in 3000 cc. water is placed, and then a solution of 1200 g. sodium hydroxide in 13000 cc. water. The valve on the inlet-tube is closed and steam is led into the container until the fluid mixture has a temperature of 60° . Then compressed air is led in and this together with the ammonia is forced over into the precipitation vessel under a pressure of 2 atmospheres. Here the stirring apparatus is set agoing, and, together with the passing air current, it brings the precipitation fluid in violent motion.

For 12 min., compressed air or steam are supplied alternately to the ammonia developer, so that the temperature here rises to 80° .

The reaction heat in the precipitation vessel is balanced by a suitable flow of cold water through the spiral cooler, so that the temperature of the precipitating mixture is kept at $61^\circ \pm 1^\circ$.

At first the aluminium nitrate solution keeps perfectly clear, but gradually — under the addition of ammonia — it becomes opaque and more viscous. Finally the content of the precipitation vessel congeals spontaneously, forming now a transparent gel. Then the ammonium supply is stopped, and the gel is evacuated into 50 liters distilled water and centrifuged in a separator. After centrifuging the sediment is washed twice, being suspended in 40 liter water in a whipping machine, and then centrifuged. The washed sediment is suspended in distilled water. The gel is filled with distilled water to make 20 liters. It contains about 1.4 % Al_2O_3 , besides the trace of NH_4NO_3 .

In its present form, the preparation is markedly thixotropic. After standing for a short time it forms a gel which on whipping turns into the sol form and then, on standing, again forms a gel. When left standing in aluminium buckets, it gradually turns into an almost perfectly clear sol, and when this is poured out into distilled water freed from CO_2 , the resulting solution is perfectly clear. If, on the other hand, the preparation is left standing in containers of resistant glass, it soon forms a gel which, after some time, gives off water and no longer makes a clear solution when it is poured out in distilled water.

The adsorptive capacity of the preparation as measured against diphtheric toxin is not affected under this conversion phenomenon but keeps unchanged even on storage for several years. But the same

rule applies to this preparation as to other active aluminium hydroxide gels: if they are stored in a wrong way — *e. g.*, in containers of ordinary bottle glass, earthenware or other vessels that may give off silicate and alkali — its adsorptive capacity will soon be lowered.

The adsorptive is assayed after the amount of diphtheria toxin which the aluminium hydroxide gel is able to adsorb from a toxin broth adjusted to pH 6 by means of hydrochloric acid. A series of adsorption tests are performed with increasing amounts of aluminium hydroxide gel against the same amount of toxin broth.

Example: 200 cc. diphtheria toxin broth is adjusted to pH 6 with hydrochloric acid and distilled water is added to make 300 cc.

The mixture is distributed on 4 centrifuged tubes, 75 cc. in each. Increasing amounts of aluminium hydroxide gel are added 2½ — 5 — 7 and 10 cc. Each portion of adsorbent is diluted with distilled water to make 50 cc.

The mixture is centrifuged, and the sediment is washed once with distilled water.

The sediment is eluted twice, each time being stirred with 40 cc. 2.4 % sodium phosphate solution (Na_2HPO_4 , 12 H_2O), and distilled water is added to the pooled centrifugates to make 100 cc. — Elutions are made of all four adsorbates in this way.

The toxin content of the elution is measured after Ramon's flocculation method, and it is calculated how many per cent of the original toxin has been adsorbed in the individual cases. The calculated values are plotted in a coordinate system with the adsorbed toxin per cent as ordinate and the employed vol. % aluminium hydroxide gel as abscissa.

(On employment of 20 vol. % of the gel described above as adsorbent for diphtheria toxin, at least 90 % of the toxin will be found in the elution.)

After storage for a considerable length of time the aluminium hydroxide gel shows tendency to conglomerate formation (when it is poured out in distilled water, lumps of gel are seen). Before using, therefore, the gel is minced, being forced through a homogenisator by a pressure of 100 atmospheres. A similar effect is obtained when the gel is whipped vigorously in a whipping machine.

Ultrafiltration.

After removal of unspecific proteins by adsorption with aluminium hydroxide gel, the antitoxin-carrying protein is present in highly diluted solution (the volume of this being about 5 times larger than the original amount of blood). As mentioned, this solution is filtered through a Seitz clearing filter. The necessary concentration of the antitoxin-carrying protein is performed rapidly and without any demonstrable loss of antitoxin by the so-called ultrafiltration through collodion membranes.

The technique of the ultrafiltration, largely based on the method given by Bechhold-König has been as follows:

The apparatus consists of kidney-shaped, two-necked balloons of porous porcelain, the so-called kidney filters. The non-filtering surface — *i. e.*, the upper part of the necks of the kidney filters — is glazed.

For impregnation with a collodion membrane the kidney filter is first placed in distilled water for 24 hours. With a slight degree of vacuum, some distilled water is sucked through the filter, which is then taken up, emptied of water and dried carefully with a towel free from loose fibers.

. The filter is then immersed cautiously in the solution of nitrocellulose in glacial acetic acid. Under the immersion of the filter care must be taken to avoid any formation of airbubbles, as such bubbles usually lead to perforation of the membrane. After finished immersion of the filter, the air is sucked out at once, and the complete vacuum is maintained for 30 sec., whereafter the cock again is opened for inlet of atmospheric air.

Now the filter is taken up — just as carefully as it was immersed. By deft turning of the filter the layer of collodion is distributed evenly over the entire surface, and the filter is at once immersed in distilled water. This results in immediate superficial coagulation of the collodion, and within a few minutes the coagulation is complete. After 15 min. the filter is quickly transferred to a large vessel with distilled water, in which it is left till its use — though at least for 24 hours — and here the strength of the membrane increases so that it will stand transfer to the filter apparatus proper and the friction arising from the stirring of the fluid to be filtered. Apart from the distilled water, nothing should come in direct contact with the membrane, which is readily destroyed by pressure of finger nails or other sharp edges.

The pore-size of the membrane depends on the nitrocellulose content of the glacial acetic acid collodion. The higher this content, the more narrow will the pores be (besides, the width of the pores can be altered by using more or less dilute acetic acid for coagulation fluid instead of distilled water). The membranes employed for the separation of the antitoxin-carrying protein are made of collodion containing 40 g. nitrocellulose per liter. (As a matter of fact, the antitoxin-carrying protein may be obtained quantitatively with membranes made of a collodion that contains 35 g. nitrocellulose per liter, but such membranes are very fragile, and hence it is preferable to use collodion containing 40 g. per liter. For other purposes in the immunochemical laboratory, where ultrafiltration may be employed with advantage, the membranes will have to be made of collodion with a higher nitrocellulose content. Thus, for ultrafiltration of diphtheria or tetanus toxin the membranes will have to be made of collodion containing 50 g. nitrocellulose per liter. For ultrafiltration of tuberculin

the membrane are made of collodion containing 70 g. nitrocellulose per liter.)

Nitrocellulose for collodion membranes is relatively easy to make in the laboratory. As a preparation of antitoxin-carrying protein often calls for a rather large amount of Nitrocellulose, and as the cost of this is relatively low, it often will be preferable to buy the nitrocellulose, which should have a nitrogen content of 11.60 %. For shipment, the nitrocellulose is moistened with alcohol, which has to be removed before using. This is done by drying the nitrocellulose in vacuum desiccator over sodium hydroxide.

Dried nitrocellulose is readily combustible and powderized, and it has a tendency to become charged electrically on which accounts it should be treated very carefully.

For solvent we employ 99 % glacial acetic acid (sp. gr. at 20°: 1.053). We prepare the solution in volumetric flasks of 3 l. The flask is filled halfway with glacial acetic acid, and then 120 g. nitrocellulose is pushed down into the flask, which then is filled $\frac{3}{4}$ with glacial acetic acid and left standing to the next day.

Now a solution of 30 g. potassium carbonate in 300 cc. glacial acetic acid is added to the contents of the flask, which then is filled to the mark with glacial acetic acid.

The flask is fastened in a machine, through which it rotates slowly (8 revolutions per min.), and this rotation is continued for about 2 weeks, whereafter the collodion is ready to use. When stored in tightly stoppered flasks, this collodion will keep unchanged for years.

Collodion showing evidence of water content — increasing viscosity or commencing opalescence — is unfit for the preparation of membranes.

The filtering capacity for a kidney filter with the fresh membrane is about 1200 cc. per hour. After a few hundreds of liters have passed through such a kidney filter, as a rule, the rate of filtration will be lowered to such an extent that the membrane has to be removed. The old membrane is spurted off by a strong jet of water under the tap, and the porcelain filter dried at 110°. The dried filter is then heated to glowing at 800°. When cooled, the filter is ready again to impregnation with a new membrane.

The ultrafiltration apparatus (Fig. 6) consists of a battery of 20 kidney filters in stellate arrangement in a round vessel with a mechanical stirrer in the center. Through an adjustable feeding-tube in its bottom, the filter vessel is supplied with the adsorbed antitoxin-containing solution from the reservoir.

The proper height of fluid in the filter vessel is adjusted by a Mariotte tube placed in the reservoir. The vacuum for the filters is provided from water pumps that are fed from a centrifugal pump, so that the same water keeps circulating in the pump system (see Fig. 7). Each battery of 20 kidney filters can handle 18—24 liters per

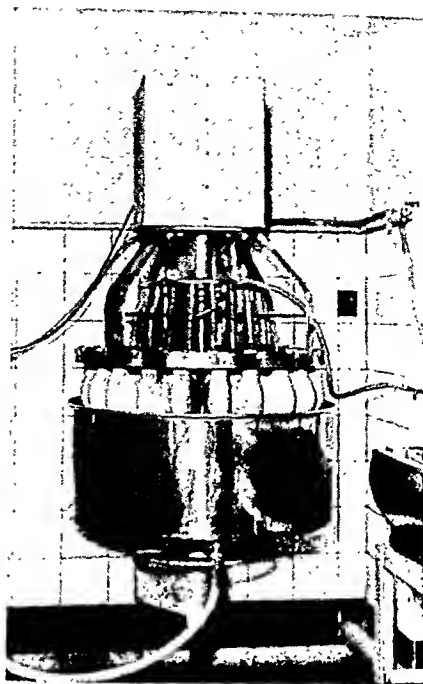


Fig. 6.
Ultrafiltration battery (see also Fig. 7).

hour. As a rule, we employ 2 batteries serially, so that 800—1000 liters are filtered in 24 hours.

During the ultrafiltration the fluid surfaces in the reservoir and the filter vessels are covered with a thin layer of toluene.

Diagrammatic Sketch of the
Ultrafiltration Apparatus.

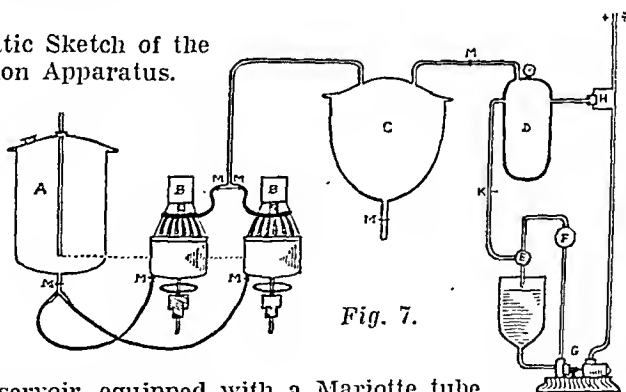


Fig. 7.

- A: Reservoir, equipped with a Mariotte tube.
- B: Ultrafilter Batteries.
- C: Receiver for collection of ultrafiltrate.
- D: Vacuum container.
- E: Water jets vacuum pump.
- F: Pressure container for water.
- G: Centrifugal pump.
- H: Relay, starting the centrifugal pump on fall in vacuum.
- K: Reverse valves.
- M: Valve.

Salting-out.

As a rule, the ultrafiltration is stopped when the amount of fluid round the filters is reduced to 50—60 liters.

Besides the antitoxin-carrying protein, the ultrafiltrate contains considerable amounts of serum albumin. Fractionation of the proteins is performed by salting-out of the antitoxin-carrying protein with ammonium sulphate. On addition of increasing amounts of ammonium sulphate to the fluid, the salting-out of the antitoxin-carrying protein commences when the ammonium sulphate concentration has reached a value corresponding to ca. 40 % saturation. When the ammonium sulphate concentration is increased to 55 % saturation, all the antitoxin-carrying protein is salted out, while the serum albumin remains in solution. In practice the salting-out of the antitoxin-carrying protein is performed as follows: the concentrated protein solution of the ultrafiltrate contains an admixture of toluene (added as antiseptic under the ultrafiltration). This toluene is readily removed by filtration through a Seitz clearing filter. Then the clear filtrate is saturated 55 % with ammonium sulphate by dissolving 290 g $(\text{NH}_4)_2\text{SO}_4$ in each liter of the fluid. Before its use for this purpose the salt had been sterilized by autoclaving. Now it is dissolved in the fluid under stirring within a few minutes. The precipitated mixture is then allowed to stand for about 1 hour, whereafter the salting product is



Fig. 8.
Filter press as constructed by the writer (see the text).

separated by filtration. In order to get the filtration performed as quickly as possible, the writer constructed a filter press with main filters of quartz filter plates and fore-filters of hard filter paper (baryte paper, 602 e. h. Schleicher & Schull (see Fig. 8).

With employment of a pressure of 0.5 atmosphere, a press with two chambers can filter 100 l. fluid in 6 hours. Then the pressure is increased to 1—1.2 atmospheres. Under this pressure the filter cake is blown through for 12 hours.

Dialysis.

The filter cake, consisting of a plastic mass is freed from its ammonium sulphate content by dialysis through cellophane tubes in running water. These tubes, which are sterilized by autoclaving under water, measure 40 mm. transversally.

The mass is placed in the tubes by a special filling machine.

This machine consists of a cylinder (15 cm. in diameter, 40 cm. in length, one end of which is provided with a nozzle, 10 cm. long, with a caliber of 18 mm.), together with a close-fitting piston that can be forced forwards under great pressure by means of a spindle.

The mass is transferred to the cylinder and then forced out through the nozzle into the dialyzing tube. This tube is cut in pieces of $1\frac{1}{2}$ m. in length, making thus throughout the length of each piece of tube a column of the mass, 18 mm. in diameter. A short glass tube (sterilized in oven) with a stopper of cotton is placed in either end of the tube; the ends of the tube are tied firmly, and the dialyzing tubes are then suspended in glass vessels — bells, 40 cm. in diameter, 60 cm. in height. The dialyzing fluid is introduced in the upper broad part of the bell as a slow and steady stream. The fluid used runs out in the bottom (that is, through the neck of the bell) which is closed with a perforated rubber stopper provided with a vertical tube that regulates the height of the fluid in the dialyzing vessel. Tap water is used for the dialysis. After dialysis for 72 hours, the tubes are free from ammonium sulphate (test with Nessler's reagent).

The solution of the antitoxin-carrying protein is evacuated from the cellophane tubes by dividing them. These tubes are used only once.

Example of the Procedure in the Preparation of Purified Antitoxin.

16 horses, immunized against tetanus, were bled twice, at an interval of 48 hours.

Each time 8 liters blood was withdrawn from each horse. Thus the starting material was

$16 \times 8 + 16 \times 8 = 256$ liters blood (corresponding to 128 l. serum, with an average titer of 500 A. U. per cc.).

1' Bleeding

$16 \times 8 = 128$ l. blood.

$16 = 22 = 352$ l. buffer.

$16 \times 450 = 7200$ cc. $n/1$ HCl.

$16 \times 1280 = 20480$ cc. bentonite sol.

Centrifuging.

Centrifugate, 450 l., free from its fibrin content
by precipitation with 4 % : 18 kg.

Bentonite sol.

Centrifuging.

Technique and propor-
tional amounts as under
1' bleeding.

To centrifugate is added 3070 cc. conc. HCl,
diluted in 50 l. dist. water (raising pH to
3.20) and 2070 cc. standard pepsin sol. 1 : 580
U. S. P. per cc., giving a pepsin content of
2 pepsin units per cc. mixture. Tp. 20°. After
1 hour, pH of 3.30 is adjusted to 3.20 by ad-
dition of 450 cc. $2/n$ HCl.

Standing for 17 hours at 20°.

Then neutralized to pH 6.5 by cautious addition
of 1640 g. NaOH dissolved in 50 l. dist.
water.

Adsorption:

1: 18 kg. aluminium hydroxide gel
2: 18 " " " "
3: 18 " " " "
4: 12 " " " "
5: 8 " " " "

Adsorption:

1: 18 kg. $Al(OH)_3$ gel
2: 18 " " "
3: 15 " " "
4: 9 " " "
5: 3 " " "

Filtration through Seitz EK.

Ultrafiltration, ca. 600 l. to 50 l.
(toluene added as antiseptic).

Salting-out with 55 % saturated ammonium
sulphate (14500 g. $(NH_4)_2SO_4$) dissolved in
50 l.

Removal of the salted out globulin by filtration
in the filter press.

Dialysis against running tap water, 72 hours
in cellophane tubes.

Dialysate: 18200 cc.

Protein content: 9 %.

Antitoxin titer: 3000 A. U. per cc.

The Finished Preparation.

After the dialysis, the solution of the antitoxin-carrying protein contains 9—10 % protein. With this protein content, as a rule, it has an antitoxin titer sufficiently high for its clinical employment in its present form.

In a few instances, however, it has been necessary to increase the protein content of the solution. By using one or more kidney filters, covered by a few per cent collodion membrane (see section »Ultrafiltration«) it is possible rapidly and without any loss of antitoxin to remove so much water that the preparation has the antitoxin titer desired.

During the various stages of the purification process the antitoxin has preserved its stability unchanged. The keeping quality of the purified antitoxins is not inferior to that of the native serum, and the purified preparations have the great advantage over native sera or antitoxin preparations made by fractionated salting-out of the serum that they keep perfectly clear without any kind of sediment even on storage for several years. The antitoxin preparations keep clear when mixed with distilled water in any proportion. When left standing, such a mixture keeps perfectly clear, without any sediment whatever.

The antitoxin titer obtained in the purified preparations depends on the titer of the blood from which the individual preparation is made.

Blood with serum titer	200— 300	A. U.	gives preparations of	1000—1200	A. U.
» » » »	400— 600	» » »	» » »	2000—3000	»
» » » »	700—1000	» » »	» » »	4000—5000	»

At the same time the yield of antitoxin is 70—80 % of the starting material. The interval between the bleeding of the horses and the end of the purification process is 8—10 days.

Now the purified antitoxins have been employed in the clinic for 10 years, given intramuscularly alone as well as by intramuscular and intravenous injection combined. They are tolerated very well by the treated patients, as the incidence of ascertained serum sickness has been considerably under 5 %.

On examination with electrophoresis and ultracentrifuging with purified antitoxin preparations are found to consist of one protein with a molecular weight of about 100000.

Although the preparation thus is a pure protein, it has been possible in various ways to split it in parts more or less rich in antitoxin.

By repeated proteolysis of the purified preparation with pepsin at pH 3.20 and following adsorption with aluminium hydroxide at pH 6.50 it was practicable in several cases to obtain an improvement of the ratio antitoxin/protein — though only at the cost of an often con-

siderable loss in antitoxin. Similar results are obtained when purified antitoxin is fractionated with ammonium sulphate. Thus, when the antitoxin is salted out in 40 % saturated ammonium sulphate and this is followed by filtration and salting-out of the filtrate by increasing the ammonium sulphate concentration to 55 % saturation, the preparation is split into a protein, relatively poor in antitoxin that is salted out at up to 40 % saturation and a protein, relatively rich in antitoxin, that is salted out between 40 and 55 % saturation.

When ammonium sulphate is added to a solution of antitoxin to 20 % saturation, and this mixture then is acidified to pH 4.20 and heated to 55° for some length of time, a smaller part of the protein, relatively poor in antitoxin, will coagulate, while the other part of the protein, relatively rich in antitoxin, remains unchanged in form in the solution.

These experiments on further purification of antitoxin preparation are theoretically interesting. Under the practical preparation of antitoxin, repeated purification has been employed but exceptionally, as it always is connected with a considerable loss of antitoxin, and as the antitoxin preparations purified through adsorption in themselves are perfectly freed from euglobulin and thus meet the most important requirement set up for an antitoxin.

Antitoxin Preparations for Veterinary Use.

Generally the same qualitative requirements are not set up for antitoxin preparations that are to be used in veterinary practice as for preparations to be employed in the human therapy. On the other hand, the lower commercial value of the former makes it essential to keep the cost of their preparation as low as possible.

A relatively good purified antitoxin is obtained, while the antitoxin content of the blood is utilized well, by employment of the following method:

The bleeding is performed under employment of hentonite as described in the section dealing with this part of the process. The clear centrifugate after the second hentonite precipitation neutralized with ammonia to pH 7.0, and then the globulin is salted out on 55 % saturation with ammonium sulphate (by solution of 290 g. $(\text{NH}_4)_2\text{SO}_4$ per liter). The salting-out product is pressed and dialyzed as described in the preceding.

Sometimes it will also be expedient to work up some weak antitoxic sera to purified antitoxin in this way.

Example: The starting material consists in 30700 cc. tetanus serum with a protein content of 10 % and an antitoxin titer of 275 A. U. per cc.

Preliminary tests: 50 cc. serum is diluted with 100 cc. in n/10 sodium acetate. This dilution is acidified with hydrochloric acid to pH 5.5, and distilled water is added to make 200 cc. Now the serum

dilution is distributed on a series of test tubes, 10 cc. in each(and dilute bentonite sol is added to the tubes in increasing amounts — as described under titration of fibrinogen (section »Bleeding«). After standing for 10 min., precipitation of fibrin appeared in tube No. 13, corresponding to an addition of 13 % bentonite sol to the serum dilution 1 + 3.

The main portion, 30650 cc., is mixed with 50 l. distilled water, in which 460 g. sodium acetate has been dissolved. The mixture is acidified to pH 5.5 with hydrochloric acid. Under vigorous stirring, the calculated amount of bentonite sol, 15940 cc., is added.

After standing for one hour the mixed precipitate is centrifuged.

The centrifugate is neutralized with ammonia to pH 7.0.

Filtration through Seitz EK. plates.

Salting-out of the filtrate (88 liters) in 55 % saturated ammonium sulphate by solution of 25.5 kg. autoclaved $(\text{NH}_4)_2\text{SO}_4$ in the fluid.

The salting-out product is separated by filtration, then pressed and dialyzed as described in the preceding sections.

The dialysate amounts to 8000 cc., with the protein content of 8.6 %, and an antitoxin titer of 850 A. U. per cc.

Antitoxins prepared with employment of this simple technique will still contain a greater or smaller amount of euglobulin, and hence it is not advisable to use these preparations in the human therapy.

Summary.

A detailed description is given of the preparation of purified antitoxins from the blood of immunized horses, besides of the apparatus and reagents used for the preparation.

The blood is defibrinated with bentonite. Blood corpuscles and fibrin are removed by centrifuging.

The solution of serum proteins is submitted to proteolysis with pepsin in acid fluid.

The unspecific protein is removed from the solution by adsorption with aluminium hydroxide gel.

The solution of antitoxin-carrying protein is concentrated by ultrafiltration, salting-out and dialysis.

The preparations thus obtained are of pure pseudoglobulin character.

The preparations are tolerated well by the patients treated with them. They keep, unchanged, and the solution remains clear, without any sediment, even on storage for years.

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SOME UNUSUAL MUCOID ORGANISMS

By Sverre Dick Henriksen.

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In a previous study of chronic respiratory diseases (1), the observation was made that it was not unusual to isolate more than one mucoid organism from the same culture. It was thought that this occurred more frequently than one would expect as a chance coincidence.

The present study was undertaken in order to seek more information as to the significance of this observation.

A number of different mucoid strains were isolated for further study. Among these, there were some unusual organisms, which are described below.

A. A mucoid strain of *Pseudomonas aeruginosa*.

A blood agar culture from a case of chronic bronchitis was found to contain a number of mucoid colonies, which looked like typical *Klebsiella* colonies. The only unusual thing about the culture was a strong smell like that of pyocyanus cultures, although no colonies of this species were found. When the culture was left on the desk for a couple of days, some of the mucoid colonies were found to dry out rapidly, leaving only a thin, shrivelled granulated film, while others remained moist and raised. On subculture, the latter colonies were found to represent the usual type of *Klebsiella*, found in such conditions, whereas the quick-drying type was found to produce a yellowish-green pigment, often a metallic lustre of the surface of the colonies, and the characteristic odor of *Pseudomonas aeruginosa*. The latter strain had the following properties:

Morphology: Short gram-negative rods like typical pyocyanus bacilli. Stained by the India ink method of Butt, Bouyngé and Joyce (2), they were found to be surrounded by somewhat irregular capsular

spaces. There also seemed to be a considerable quantity of intercellular material, probably free capsular substance. The rods were non-motile.

Colonies: 24 hours old colonies are indistinguishable from *Klebsiella* colonies, raised, strongly mucoid, of a thin, syrupy consistency. After 48 hours at 37 C or 3—4 days on the desk the colonies shrink to thin granulated films, usually with a metallic lustre. (Photo no. 1).

Subcultures from young, mucoid colonies usually gave a pure growth of the same type of colony, whereas subculture from older colonies gave a mixture of mucoid and non-mucoid colonies. Thus the strain had a marked tendency to produce non-mucoid mutants, this tendency being most marked at 37 C, less at lower temperatures. The non-mucoid colonies were of two types, one just like the usual pyocyaneus colony, the other smaller, smooth, raised with a navel-shaped depression on the top. This latter type was unstable and changed into the former type on subculture.

By always selecting strongly mucoid colonies for subculture, it has been possible to maintain the strain in the mucoid state for about 2 years so far.

Cultural and biochemical properties: Good growth on all the usual media between room temperature and 37 C. In broth even turbidity and a surface pellicle, later a viscid sediment. Poor growth under a vaseline seal.

Potato: thick brownish growth with a metallic lustre.

A yellowish green pigment is produced on all media.

Fermentation: Weak acid production from glucose. Maltose, lactose sucrose and mannitol are not fermented.

Milk: Coagulated in 48 hours, later slowly peptonized.

Gelatin: Sacculiform, later complete, liquefaction.

Serum slants: No liquefaction.

Nitrates are reduced to nitrites, but indol is not produced in peptone broth.

A solution of dimethyl paraphenylene diamine gives a positive oxydase reaction.

H₂S is produced in lead acetate agar.

Classification: The strain must be considered as a mucoid strain of *P. aeruginosa*.

Comment. Mucoid strains of *P. aeruginosa* are rare in human material. They are also stated to be rare elsewhere in nature. Such strains have been described previously by Sonnenschein (3), by Dahr and Kolb (4) and possibly by Pottien (3). Reid, Harris, Naghski and Gatchell have studied the dissociation of this species and have seen the mucoid form (5). The original reports on these forms were not available.

The simultaneous presence of this strain and a typical strain of *Klebsiella* in the same sample should be noted.

B. A mucoid strain of Staphylococcus aureus.

A throat swab from a case of chronic rhino-pharyngitis yielded practically pure growth of a strongly mucoid organism with white or ivory colored colonies. It was found to consist of gram-positive micrococci.

Morphology: Gram-positive cocci of the size, shape and arrangement of staphylococci. Stained by the India ink method (2) they were found to be surrounded by distinct capsular spaces.

Colonies: Large, 3—4 mm after 24 hours, moderately raised, very moist and confluent, of a thin, not markedly viscid consistency. The pigment varied in different cultures from pure white to pale yellow, and usually was concentrated in the centre of the colony, whereas the periphery was colorless or greyish, transparent. The yellow color was most marked in cultures cultivated at room temperature. The strain has remained quite stable in the mucoid state for about 2 years, with no tendency to dissociate (Photo no. 2).

Cultural and biochemical properties: Good growth on all the usual media. Broth cultures showed even, dense turbidity.

Fermentation: Acid was produced from glucose, maltose, lactose, sucrose, mannitol, dulcitol, trehalose, levulose, dextrin and inulin, but not from salicin, aesculin, rhamnose, arabinose, xylose, sorbitol or starch.

Gelatin: Sacculiform liquefaction from the 3rd or 4th day.

Milk: coagulated within 14 days.

Nitrates are reduced to nitrites but neither indol nor H_2S are produced.

No distinct hemolysis on blood agar.

Crystal violet agar (1:300000): growth with violet colonies which later turned yellow in the centre.

0.017 % bromothymol blue agar: good growth.

Plasma coagulase test: positive in $1\frac{1}{2}$ hours.

Mice were killed by intraperitoneal injection of 0.5 ml 24 hour broth culture, but not by 0.05 ml.

Classification: This strain gives the reactions of the pathogenic type of *Staphylococcus aureus*: fermentation of mannitol, violet growth on crystal violet agar, growth on bromothymol blue agar, and plasma coagulase test. The production of pigment was weak and irregular, but sufficient to show that it must be the aureus variety.

Comment. Mucoid forms of *Staph. aureus* have been described by Sonnenschein (3), Oesterle (6) and by Gilbert (7). All strains produced a strong yellow pigment, and the two latter strains were pathogenic to mice.

Bigger, Boland and O'Meara (8) studied the dissociation of staphylococci and found some »viscid« forms, which, however, seemed to be more closely related to the R-form than to the M-form. Apparently they were not encapsulated.

Nothing seems to be known as to the cause of the mucoid transformation.

C. A mucoid diplobacillus and an unusual strain of Klebsiella.

A nose swab from a case of chronic rhino-pharyngitis, among other organisms, gave growth of a pneumococcus type III and some fairly large, dry colonies with a navel-shaped depression. After the culture had been left on the desk for some days, an additional type of colony had appeared. This was a strongly mucoid colony of the *Klebsiella* type.

Subculture at 37 C in an electric incubator gave extremely poor, dry growth of both the navel-shaped colony and the mucoid one. At 30 C, however, both colonies gave a luxuriant, strongly mucoid growth, indistinguishable from that of *Klebsiella*. Further study showed that the navel-shaped type of colony conformed with *Klebsiella* in all respects except its unwillingness to grow at 37 C under usual conditions, whereas the mucoid colony from the primary culture was found to be a diplobacillus, the properties of which are reported below.

Both these strains were peculiar in their demand for a humid atmosphere. When cultivated at 37 C in the incubator, the *Klebsiella* only produced a very meager, dry growth on the part of the plate where the inoculum had been deposited, and the diplobacillus either failed to grow altogether, or produced an extremely meager, dry growth. When films were prepared from such growth, both strains showed extreme pleomorphism, with large swollen rods and pale »ghost cells«. Large »spherical bodies« were also produced, and other bizarre forms. Dumbbell shaped cells were frequent in the diplobacillus. (Photos nos. 3—9).

When the same strains were cultivated at 37 C in a closed jar, containing water, they always gave luxuriant, strongly mucoid growth, which showed no pleomorphism on microscopical examination. Similar growth was obtained in an incubator at 30 C or on the desk. The pleomorphism could be reproduced at will by cultivating at 37 C in a dry atmosphere. (Photos nos. 3—9).

None of the other mucoid organisms studied have shown this extreme sensitiveness to drying, nor similar pleomorphism.

Apart from this peculiarity, the strain of *Klebsiella* behaved as most other strains, and there is no need to describe its properties in detail.

The mucoid diplobacillus had the following properties:

Morphology: When grown in a humid atmosphere, the strain produced short, plump rods with a marked tendency to appear in pairs or in short chains. Grown at 37 C in a dry atmosphere it showed the pleomorphism described above.

The rods were non-motile, and were surrounded by large capsular spaces in India ink preparations. In the pleomorphic organisms the capsules still seemed to be present but very much reduced in size.

Cultural and biochemical properties: Luxuriant, mucoid growth on all solid media, if a humid atmosphere was provided. Very poor or no growth on all media in a dry atmosphere at 37 C. The colonies, when drying was prevented, were large, raised, confluent, moist, moderately viscid, just like most strains of *Klebsiella*.

In liquid media growth was very poor at 37 C, better at 30 C, both in plain and rich media.

No carbohydrates were fermented, neither indol nor H_2S produced and neither gelatin nor serum liquefied.

Nitrates were reduced to nitrites, and a positive oxydase reaction was obtained with dimethyl paraphenylenediamine.

Mice were killed by intraperitoneal injection of 500 million and 50 million organisms, but not by smaller doses.

Classification: This strain does not conform with any species listed in Bergey's manual, nor with any described in the available literature. In a recent study (9), however, some diplo-bacilli from the genito-urinary tract were described, which show a marked similarity with this strain. Such strains were also found in the respiratory tract. These diplobacilli were not mucoid, but otherwise had all the properties of this mucoid strain. They were thought to be related to *H. duplex*, and to represent a new species of diplobacilli.

It seems that the mucoid strain described above must be the mucoid form of this species, the classification of which has been discussed previously (9).

Comment. This is another case where two different mucoid gram-negative rods were isolated from the same case of chronic inflammation of a mucous membrane. Both strains grew with exactly the same type of mucoid colony, showed the same sensitiveness to drying, and a similar pleomorphism, when exposed to a dry atmosphere at 37 C. Such behaviour is unusual in *Klebsiella*. None of the other strains of *Klebsiella* studied were sensitive to drying. In the diplobacillus, on the other hand, it may be the normal behaviour. This type of diplobacillus has been shown to be unwilling to grow in a dry atmosphere at 37 C. But none of the strains, described previously, were mucoid. Thus it may seem as if these two strains, growing together on the same membrane, had borrowed some characteristics from each other.

It may be wise, however, to consider this just a set of unusual coincidences, in the absence of more tangible evidence.

It is interesting to note that the mucoid diplobacillus was pathogenic to mice, whereas the non-mucoid strains studied previously, were non-pathogenic even in large doses.

Summary.

Some organisms, rarely seen in the mucoid stage, are described. One was a mucoid strain of *Pseudomonas aeruginosa*, found together with a strain of *Klebsiella*.

The second was a mucoid *Staphylococcus aureus*.

The third and fourth strains, isolated from the same case, were a strain of *Klebsiella* and a mucoid diplobacillus. The colonies of these strains were indistinguishable from each other, and they both were very sensitive to a dry atmosphere at 37 C. They showed a marked pleomorphism, characterized by large, swollen cells and spherical bodies, when incubated at 37 C in a dry atmosphere.

The mucoid diplobacillus was pathogenic to mice, whereas the smooth stage of the same organism has been found to be non-pathogenic.

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Photo no. 1.
Mucoid strain of *P.*
aeruginosa $\times 5$.



Photo no. 2.
Mucoid *Staphylococcus*
aureus. $\times 4$.



Photo no. 3.
Klebsiella, incubated in
moist atmosphere.
 $\times 4$.



Photo no. 4.
Diplobacillus, incubated
in moist atmosphere.
 $\times 4$.



Photo no. 5.
Klebsiella, humid ath-
mosphere. India ink
preparation. $\times 1850$.



Photo no. 6.
Diplobacillus, humid
atmosphere. India ink
preparation. $\times 1850$.

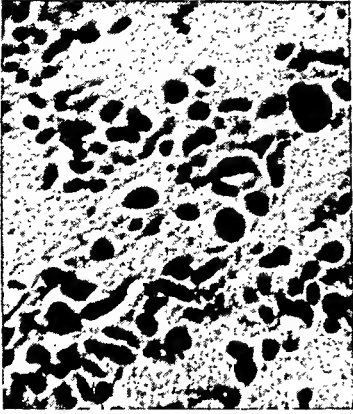


Photo no. 7.

Klebsiella. Dry atmosphere at 37 C. Pleomorphic organisms with large swollen cells. Gram's stain. $\times 1100$.



Photo no. 8.

Diplobacillus. Dry atmosphere. Pleomorphic organisms. Gram's stain. $\times 1850$.

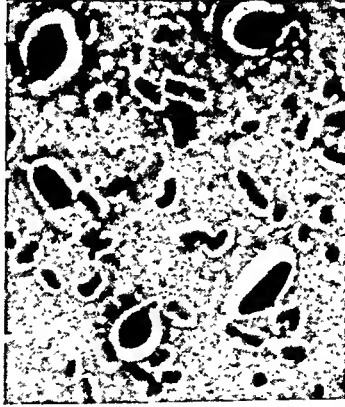


Photo no. 9.

Klebsiella. Dry atmosphere at 37 C. India ink preparation. Pleomorphic organisms with irregular capsules. $\times 1850$.

SEROLOGICAL CROSS-REACTIONS BETWEEN UNRELATED MUCOID ORGANISMS

By *Sverre Dick Henriksen.*

(Received for publication November 28th, 1947).

In previous papers (1, 2) it has been shown that two or more mucoid organisms can sometimes be isolated from the same patient. This seems to be true particularly in certain chronic diseases of the mucous membranes, which practically constantly give growth of microbes classified as *Klebsiella*.

Several explanations might be suggested to account for this fact. 1. To start with the simplest explanation, it might be a mere chance coincidence. Study of a large material, collected from such conditions and from normal persons, would be necessary to prove or disprove this hypothesis.

2. It might be due to some selective factor, favoring the establishment of mucoid organisms on the mucous membranes, e. g. some agent inhibiting the growth of certain organisms in the smooth, but not in the mucoid state. The predominance of *Klebsiella* strains in such conditions could be explained by assuming a higher mutation rate in the bacteria which give rise to the *Klebsiella* strains (possibly *Escherichia*), than in other organisms. Only bacteria with a comparatively high mutation rate might have a reasonable chance of establishing themselves in the mucoid state, before being suppressed by the selective agent.

3. The studies of Avery et al. (3, 4, 5) and of Boivin et al. (6, 7) on type transformation suggest the possibility of transfer of gene material between two organisms growing in the same place. So far, type transformation has only been observed within the same species. Whether it is possible between more distantly related or unrelated species is unknown.

These three hypotheses would not necessarily exclude each other.

An antigenic study of mucoid organisms, isolated from the same patient, might possibly give some indication as to which hypothesis was most likely in a given case.

Material and methods.

The strains studied include the unusual strains described in the preceding paper (2), namely:

A mucoid strain of <i>Pseudomonas aeruginosa</i> ,	— Strain P-752
A <i>Klebsiella</i> strains for the same patient,	— Strain K-752
A mucoid diplobacillus,	— Strain D-H4
A <i>Klebsiella</i> strain from the same patient,	— Strain K-H4
A mucoid <i>Staphylococcus aureus</i> ,	— Strain LKA

In addition, 25 other mucoid gram-negative rods, collected from various sources, were included in the study. These strains were isolated from the following sources:

Chronic rhino-pharyngitis (incl. ozaena)	7 strains
Asthma and chronic bronchitis	8 strains
Bronchopneumonia	2 strains
Pleurisy	1 strain
Liver abscess	1 strain
Purulent infections	2 strains
Urinary infections	3 strains

The cultural and biochemical reactions of these strains were studied in some detail, in order to get an idea as to which types were prevalent.

Serological methods.

Immunization: Rabbits were immunized by intravenous injections of formalin-killed bacterial suspensions on the first 5 days of each week for 3—4 weeks. Dosage started about 500 million organisms and increased gradually up to about 5000 million. Additional courses were given if test bleedings were found to be of insufficient titer. Serological tests: Attempts to utilize the agglutination reaction were not successful. Satisfactory titers were not obtained. The complement fixation reaction gave more promising results, but considerable work was necessary to find the most satisfactory method. The first attempts were made with incubation in a water bath at 37 C for 30' to 1 hour. Whereas homologous reactions usually were satisfactory with an incubation time of 1 hour or even 45', some cross-reactions gave variable results, and after incubation for 30', even the homologous reactions became inconsistent. It appeared as if the reaction with *Klebsiella* antigens was somewhat sluggish, and required considerable time to be completed. This may possibly be due to the high viscosity of the antigens.

Incubation at 4 C for 20 hours gave more satisfactory and reproducible results. The technique finally adopted was the following: Two-fold dilutions of inactivated immune serum were made up in 0.2 ml volumes, starting with 0.1 ml of serum (the dilutions were actually prepared in larger volumes, to obtain greater accuracy, and 0.2 ml volumes pipetted into a series of tubes).

Antigen: 0.2 ml of saline suspensions of heat-killed organisms, standardized to contain between 1000 and 2000 million cells per ml. Complement: 0.2 ml volumes, containing 2 hemolytic units (the unit determined as the smallest quantity necessary to give complete hemolysis with 0.4 ml 1.5 % sheep blood corpuscles, sensitized with 4 units of hemolytic amboceptor).

Incubation at 4 C for 20 hours. After incubation, addition of 0.4 ml of sensitized sheep blood corpuscles, and renewed incubation at 37 C. The tests were read after 15', and again after complete sedimentation of the blood corpuscles at room temperature.

Titers are given as the serum dilution in the total reaction mixture of 0.6 ml, in the last tube that gives at least 50 % fixation (i. e. less than 50 % hemolysis). Thus the serum dose 0.1 ml would correspond to the titer 1:6.

Experimental.

A. The properties of the Klebsiella strains.

Type of colony: Two different types of colonies could be distinguished with comparative ease. The predominant type (21 strains) was large, moderately raised, confluent, of a thin, syrupy, not very viscid consistency. The second type (2 strains) was larger, domed, of a firmer, more viscid consistency, like a sticky jelly. 4 strains differed slightly from the others, the colonies, while resembling the predominant type, were smaller, and one of them showed a marked tendency to split out smooth mutants. 3 of these strains were isolated from urinary infections. These strains differed from the others in other respects also, and were considered as mucoid strains of *E. coli*, rather than *Klebsiella*, that is if there is any real difference between the two.

Fermentation reactions: On bromothymol blue lactose agar only 4 strains produced an acid reactions. 23 strains grew with blue colonies. All strains produced acid from glucose, maltose and mannitol. Lactose was fermented by all but 4 strains after 24 hours to 7 days, and sucrose was fermented by 4 strains only. Only 8 of the strains produced gas, 3 urinary strains produced indol, and 3 of the strains, two of which were urinary, were weakly motile. 4 strains produced H_2S .

All strains gave a positive methyl red test, and all, except one, a negative Voges Proskauer test.

Thus a similar variation was found in this material as in most previous investigations of this group.

B. Complement fixation reactions of *Klebsiella* strains.

Complement fixation tests were set up in two different anti-*Klebsiella* immune sera (serum anti-K-H4 and serum anti-K-752), with 25 different strains as antigens. The homologous titer of both sera was 1:192. 18 strains gave reaction up to the homologous titer, within the experimental error. All variations on repetition of the tests, were within one tube below or above the homologous titer. 7 strains gave negative reactions, including 3 strains from urinary infections, the two strains with the domed colony, 1 ozaena strain and 1 bronchopneumonia strain.

Thus the majority of mucoid strains isolated from various chronic conditions of the mucous membranes seem to belong to the same serotype.

Table 4.

Cross-reacting antibodies to mucoid organisms demonstrated by fixation of complement. Antibody titers with constant antigen dose.

Immune sera	Antigens				
	<i>Klebsiella</i> K-752	<i>Klebsiella</i> K-H4	<i>Diplobacillus</i> D-H4	<i>Staphylococcus</i> , LKA	<i>P. aeruginosa</i> P-752
Anti K-752	192	192	192	0 §	0
Anti K-H4	96	192	192	24 §	0
Anti D-H4	48	48	3072	96	—
Anti LKA	0	0	192	192	—
Anti P-752	0	0	—	—	768

—: reaction not carried out.

§: These reactions gave variable results.

C. Cross-reactions between *Klebsiella* and other mucoid organisms.

Immune sera were prepared as described above against the mucoid strain of *P. aeruginosa* (anti-P-752), the mucoid diplobacillus (anti-D-H4) and the mucoid *Staphylococcus* (anti-LKA).

Complement fixation tests were set up with different antigens in these immune sera and the two anti-*Klebsiella* sera. All tests were repeated on several occasions, and the results found to be consistent. All variations seemed to be within the experimental error. The results are presented in table I.

The table shows that the diplobacillus cross-reacts in the two *Klebsiella* immune sera up to the homologous titer, whereas the *Klebsiella* antigens only show moderate cross-reactions in the anti-diplobacillus immune serum. Surprisingly enough, the mucoid *staphylococcus* shows marked antigenic similarity with the diplobacillus. The slight, and somewhat irregular, cross-reactions in anti-*Klebsiella* im-

mune sera with the staphylococcus antigen may indicate a slight antigenic similarity, but the reactions were not entirely convincing.

The strain of *P. aeruginosa* showed no cross-reactions with the *Klebsiella*, isolated from the same patient. This negative reaction was confirmed by tests with crude, but probably protein-free, capsular polysaccharides, prepared from the two strains. These polysaccharides gave homologous precipitation and complement fixation only.

The cross-reactions between the diplobacillus and the *Klebsiella* from the same patient are of particular interest in this study. The question arises whether these, strong, but not complete, cross-reactions would be consistent with the 3rd hypothesis, suggested above, i. e. whether the cross-reactions might possibly be due to identical capsular polysaccharides. As it is unknown to what extent the capsular and somatic antigens contribute to the reactions in these cases, more detailed antigen analysis would be necessary to answer the question.

The cross-reaction between the diplobacillus and the mucoid staphylococcus, although interesting, was not thought to be equally pertinent to this study, and was left for future consideration.

D. Preparation and serological assay of capsular polysaccharides.

In view of the fact that the *Klebsiella*, strain K-H4, gave complete cross-reactions with a majority of *Klebsiella* strains, it was thought most likely that it was a representative of Julianelle's group A. The method used by Goebel and Avery (8) for the preparation of the capsular polysaccharide of this group was consequently chosen as the one most likely to succeed.

30 agar plates each were inoculated with the two strains. After 48 hours at 30 C (under which conditions a luxuriant mucoid growth was always obtained), the growth was harvested and suspended in 300 ml of water, after which the directions given in (8) were followed as closely as possible.

The yields of capsular polysaccharide were 0.45 G from strain K-H4 and 0.1115 G of strain D-H4. The yields were insufficient for a complete chemical analysis, but some properties were examined. Both polysaccharides were greyish-white amorphous substances, which were fairly easily soluble in water (more easily in dilute alkali). Watery solutions were slightly opalescent, very viscous and gave a rather high acidity. About 2 % solutions of the polysaccharides had a pH of 2.22 for K-H4 and 2.37 for D-H4. The biuret reaction was negative in fairly concentrated solution, and the Molisch test positive. One of the solutions reduced Fehling's solution (D-H4), the other only after acid hydrolysis. Some other properties are shown in table II. For comparison the properties of Friedlander polysaccharides, as stated in (8) and (9) are shown in the same table.

It seems obvious that the degree of purification in these experiments was less satisfactory than in the studies referred to above. It

Table 2.

Properties of polysaccharides K-H4 and D-H4, compared with Friedlander types A, B and C polysaccharides, according to (8) and (9).

Origin of polysaccharide	Acid equivalent	(α) D	Nitrogen	Highest dilution giving precipitate in immune serum
Type A	430—445	—100° to —105°	0	1:2×10 ⁵
Type B	670—722	+100° to +102.5°	0	1:2×10 ⁵
Type C	575—681	+90° to +101°	0	1:2×10 ⁵
Strain K-H4	604	+62°§	1.50%	1:2×10 ⁵ §
Strain D-H4	578	+10°§	2.60%	1:2×10 ⁵ §

§: measured at pH 7.

§: Slight trace of precipitate after centrifugation with this dilution.

is felt that the small quantity of nitrogen probably represents some impurity. Serological tests, however, indicated that the degree of purification might be sufficient for the purpose of this study.

It is reasonably certain, when comparing the optical rotations of the different polysaccharides, that the one prepared from strain K-H4 is different from that of Julianclle's group A, contrary to expectation. It may however be identical with that of group B or group C. Studies of the reactions of autentic type-strains will be necessary to decide which. At any rate the sero-type, found to predominate in this collection of strains, seems to be another one than in Julianclle's material (10).

The differences in the optical rotations of the polysaccharides from strain K-H4 (s-K-H4) and from strain D-H4 (s-D-H4) suggest that these polysaccharides are different, although the purity of the preparations may not allow definite conclusions.

Precipitation tests with various dilutions of the two polysaccharides in the two immune sera, gave homologous precipitation only. Each preparation reacted with homologous serum up to a dilution of $1:2 \times 10^6$.

Complement fixation tests gave entirely different results, as shown in table III.

Both preparations produced complete fixation with the homologous serum up to dilution $1:3.2 \times 10^6$, whereas cross-reactions did not go further than to dilution 1:3200 and 1:12800 respectively.

Thus the cross-reactions between the two organisms seem to be due, in part at least, to related capsular polysaccharides.

Some absorption experiments were carried out, in order to find out whether absorption of a serum with the homologous polysaccha-

Table 3.

Complement fixation with capsular polysaccharides. Constant dose of serum (0.04 ml) and four-fold dilutions of antigen.

Immune serum	Highest reactive antigen dilution	
	Antigen S-K-H4	Antigen S-D-H4
Anti K-H4	$1:3.2 \times 10^5$	1:12800
Anti K-752	$1:3.2 \times 10^6$	1:12800
Anti D-H4	1:3200	$1:3.2 \times 10^5$

ride would remove all of the cross-reactive antibody. The results, which were unsatisfactory, are presented in table IV.

Table 4.

Anticomplementary activity and specific combining power of immune sera, absorbed with capsular polysaccharides. ..

Immune serum Absorbed with		Anticomplementary titer	Specific combining power	
Anti K-H4	s-K-H4 1:4000	1:96	Antigen K-H4 1:192	Antigen D-H4 1:384
Anti D-H4	s-D-H4 1:4000	1:96	1:192	1:3072
Anti K-H4	s-K-H4 1:10000	1:24	1:384	1:384
Anti D-H4	s-D-H4 1:10000	1:24	1:48	1:3072

Absorption was carried out with equal volumes of serum and of the antigen dilution stated in the table. The bacterial antigens were standardized to contain 1000 million cells per ml.

In the first attempt, absorption was made with a quantity of polysaccharide, sufficient to give a slight antigen excess, as determined in precipitation tests. The absorbed sera became extremely anticomplementary, possibly indicating that the precipitation had been incomplete, and that small soluble antigen-antibody complexes remained in the supernatants. It was thought that this might have been due to a too large antigen excess, and a dose from the equivalence zone was chosen for the next attempt. After absorption with this quantity, allowing several days in the refrigerator for completion of the reaction, the supernatants were less anticomplementary than in the first experiment, but no reduction of the titers, either homologous or heterologous, could be detected.

Thus attempts to absorb the cross-reacting antibody were unsuccessful.

cessful. Thus it is possible that somatic antigens may have played a rôle in the cross-reactions, but it is perhaps more likely that the failure may be due, either to failure in selecting a proper dose for absorption, or to some degree of degradation of the polysaccharides in the process of purification, which has reduced their specific combining power. This would also account for the fact that cross-reactions with polysaccharides were weaker than one might have expected, after the strong reactions with the complete bacterial antigens.

Discussion.

It appears from the results that the predominant sero-type in this material, isolated mainly from mild chronic infections like ozaena and chronic bronchitis is different from the type found to predominate in Julianelle's material. It is possible that differences in the selection of the strains may account for this difference. Julianelle's strains to a larger extent originated in more severe infections, such as Friedlander pneumonia, a disease which seems to be rare in Norway. This question must be left open for future studies.

The complement fixation reaction seems to be well suited for the study of mucoid organisms, if sufficient incubation time is allowed for completion of the reaction. The reaction seems to be more sensitive when it is a question of detecting minor, cross-reacting antigenic components, than either the agglutination or precipitation reactions.

The failure of the precipitation technique to demonstrate the cross-reacting antigens in this study may possibly be due to the formation of small, soluble antigen-antibody complexes, which are too small to precipitate, but large enough to fix complement.

No decision could be achieved as to whether the cross-reaction between a *Klebsiella* and a mucoid diplobacillus from the same patient was due to cross-reacting capsular polysaccharides only. Ample evidence was obtained, however, to show that the two polysaccharides were not identical.

There is thus no evidence to indicate that the peculiar similarities between these two strains are anything more than coincidences. The results are further confirmation of the observation that cross-reactions between polysaccharides of different origin are fairly common.

The cross-reaction between a mucoid diplobacillus and a mucoid staphylococcus is of some interest, but a complete analysis of this reaction can not be offered at the present time.

Summary.

The predominating sero-type among a number of *Klebsiella* strains, isolated from various mild, chronic infections, appears to be some other type than Julianelle's type A.

A mucoid strain of *P. aeruginosa* was found to be serologically unrelated to a *Klebsiella*, isolated from the same patient.

A mucoid diplobacillus showed a marked cross-reaction with a *Klebsiella* from the same patient, and also with a mucoid *Staphylococcus aureus* from a different patient.

The former cross-reaction was found to be due, in part at least, to cross-reacting, but different capsular polysaccharides.

The complement-fixation seems to be more sensitive than other reactions in detecting antibodies against mucoid organisms of these types, and particularly, cross-reacting antibodies.

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ON HAEMAGGLUTINATION BY ESCHERICHIA COLI

By *F. Kauffmann.*

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Having worked on the serology of the Coli group during the past few years, especially on the distribution of the haemolytic strains among the various serotypes, it became natural to include the haemagglutination of the Coli bacteria in these investigations. Our previous experiments had made clear the fact that haemolytic Coli strains particularly often range among certain serotypes of the O-groups 4 and 6, whereas not one strain of O-groups 8 and 9 cause haemolysis.

As long ago as in 1902 R. Kraus and St. Ludwig reported on haemagglutination by bacteria. They demonstrated that a *Vibrio* strain (Paris) and a strain of *Staphylococcus aureus* haemolyticus agglutinated rabbit erythrocytes in test-tubes, whereas typhoid bacilli caused no agglutination. The authors gave no indication of whether Coli strains were tested in this respect.

Afterwards, in 1908, G. Guyot published further studies of bacterial haemagglutination and established the fact that many Coli strains possess the property of agglutinating the erythrocytes of various animals. He employed slide-agglutination and a 5 % suspension of erythrocytes in saline. He showed that formalin-killed bacteria retain their agglutinating property.

In 1943 L. Rosenthal wrote on the »Agglutinating properties of *Escherichia coli*«, and confirmed and amplified Guyot's results. For example, he demonstrated that heating for two minutes at 100° C. destroyed the agglutinability of the culture, whereas alcohol treatment left it unaffected. Of 70 Coli strains isolated from faeces and tested, 12 strains agglutinated human erythrocytes.

Own Investigations.

The tests were made with living Coli cultures from 20-hour agar plates. The culture was stirred direct with a needle into a drop of 10 % blood (in saline solution) on a slide. After the slide had been tilted back and forth for about thirty seconds the reaction was read with the naked eye; in doubtful cases a lens (8x) was used.

The blood used was from man, guinea-pig, horse, rabbit and chick.

Chick blood was chosen for the reason that experiments with viruses (Hirst test) have shown that this kind of blood is particularly suitable for haemagglutination. Some tentative tests showed that it is immaterial whether one employs 5 % or 10 % blood and whether the blood is washed or not.

In making the tests, each strain was tried against all five kinds of blood at once. A total of 112 Coli strains of known antigenic structure and known haemolytic property were tested. They represented the first 25 O-groups, with particular reference to O-groups 2, 4, 6, 8 and 9. Of the 112 strains, 34 gave no agglutination in the five kinds of blood tested, and 94 strains were not haemolytic.

Thus it was found, in complete accord with the results published in the literature, that Coli bacteria possessed of haemagglutinating properties are of frequent occurrence and that haemagglutination does not run parallel with the haemolytic property. Whereas almost all haemolytic strains (there was one exception) were haemagglutinating, many strains not producing haemolysis gave strong haemagglutination. In other words, the haemagglutinating property is more frequent than the haemolytic and is distributed — though not regularly — among all O-groups so far tested.

On this matter of regularity, the point is that O-groups 4 and 6 were outstanding, giving haemagglutination particularly often, whereas O-groups 8 and 9 possessed this property much less frequently. In the table will be found the results of tests with 14 strains of O-groups 4 and 6 and 14 strains of O-group 9. As twelve strains of O-group 8 behaved in the same manner as the strains of O-group 9, and eleven strains of O-group 2 as those of O-groups 4 and 6, I have omitted to show these results in tabular form.

The table shows that of 14 strains from O-groups 4 and 6, 12 caused haemagglutination, at least in one kind of blood but usually in several or all the samples tested. Of 14 strains from O-group 9, only 5 caused haemagglutination, and in most cases it was only weak. The strongest reactions were obtained with guinea-pig blood, followed by chick and horse blood, whereas rabbit and human blood agglutinated less strongly and less often. In other words, agglutination did not always occur in all five blood samples, human blood especially failing in some instances. Therefore, if it is the intention to test a large number of Coli strains for their haemagglutinating property, it will in future suffice to work with guinea-pig and human blood, the two extremes.

The difference between O-groups 4 + 6 on the one hand and O-group 9 on the other was most evident in the case of human blood, for 9 of 14 strains from the former group (4 + 6) gave a positive reaction, whereas only 2 out of 14 strains of the latter group (9) reacted positively. When chick blood was used, a total of 12 positive reactions was obtained in groups 4 + 6, but in group 9 only 4. Of 9

haemolytic strains in group 4 and 6, 8 were also haemagglutinating.

Whether or not the strains were possessed of L-antigen did not affect their haemagglutinating property, as both forms, L plus and L minus, of strain U 5/41 (O-group 1) agglutinated guinea-pig blood strongly, the other kinds of blood only weakly.

The haemagglutinating property was not destroyed by heating a bacterial suspension (in NaCl) at 70° C. for one hour in a waterbath, although this treatment killed the bacteria. But bacteria heated for 5 minutes at 100° C. no longer caused haemagglutination. Treating with 1 % formalin, though killing the bacteria, did not affect the haemagglutinating property.

Table 1.
Haemagglutination by Escherichia coli.

Strain	Antigen			Haemo- lysis	Erythrocytes from				
	O	K	H		Hen	Rabbit	Horse	Guinea-pig	Man
U 4/41	4	3 L	5	+	+	+	+	+	+
A 104a	4	3 L	.	+	—	—	—	—	—
Bi 7457/41	4	6 L	5	+	+	+	+	+	+
A 93a	4	12 L	5	+	+	(+)	(+)	+	+
Su 65/42	4	12 L	.	+	+	(+)	(+)	+	+
A 77b	4	52 L	4	—	+	+	+	+	+
A 103a	4	52 L	.	—	(+)	(+)	+	+	—
Bi 7458/41	6	2 L	1	+	+	+	+	+	+
Su 4344/41	6	13 L	1	+	+	+	+	+	+
PA 151	6	13 L	.	—	(+)	—	—	—	—
K 14b	6	.	10	+	+	+	+	+	(+)
F 8316/41	6	15 L	16	—	(+)	(+)	(+)	+	—
PA 236	6	53 L	.	+	+	+	+	+	+
A 12b	6	54 L	10	—	—	—	—	—	—
Bi 316/42	9	9 L	12	—	+	+	+	+	(+)
A 354b	9	26 A	10	—	—	—	—	—	—
Bi 449/42	9	26 A	.	—	—	—	—	—	—
K 14a	9	28 A	.	—	—	—	—	—	—
Bi 161/42	9	29 A	.	—	—	—	—	—	—
A 43c	9	30 A	19	—	—	—	(+)	(+)	—
A 292a	9	31 A	4	—	—	—	—	—	—
Su 3973/41	9	31 A	.	—	—	—	—	—	—
A 95b	9	32 A	5	—	+	+	+	+	(+)
H 36	9	32 A	10	—	+	(+)	(+)	(+)	—
A 45a	9	32 A	.	—	(+)	(+)	(+)	(+)	—
Ap. 189	9	33 A	.	—	—	—	—	—	—
A 55a	9	34 A	.	—	—	—	—	—	—
F 294a	9	36 L	.	—	—	—	—	—	—

Explanation of signs: + = positive, (+) = weak positive, — = negative.
Haemolysis = tested by horse erythrocytes.

Discussion.

The object of this investigation was solely to ascertain the distribution of the haemagglutinating Coli strains among the more important Coli O-groups and types, and therefore I refrained from fundamental examinations of the nature of haemagglutination (apart from the resistance test just referred to).

These results have helped to add one more indication to the already known differences between various O-groups and types. The special position occupied by O-groups 4 + 6 on the one hand, and O-groups 8 + 9 on the other, also becomes distinctly evident when haemagglutination is taken into consideration. Whereas haemolysis and haemagglutination are very frequent within O-groups 4 + 6, haemolysis is completely absent in O-groups 8 + 9 and haemagglutination is relatively rare. As moreover these two groups (4 + 6 and 8 + 9) according to Ewertsen and Sjöstedt's results also differ by the fact that the occurrence of toxic and necrotizing strains are much more frequent in the former group (4 + 6) than in the latter (8 + 9), the difference between them is definitely established.

I must specially point out that not all types within one O-group behave in the same manner, and therefore the O-group determination alone will not suffice to solve Coli problems; it must be supplemented with a type diagnosis.

On the basis of all results so far (results which I have recently gathered into a Coli-review) we may take it for granted that most types within O-groups 4 and 6 are possessed of higher pathogenicity than the types within O-groups 8 and 9. The main differences may be given schematically as follows:

Differences between types of the O-groups.

4 + 6	8 + 9
1. Frequently haemolytic	1. Non-haemolytic
2. Frequently haemagglutinating	2. Rarely haemagglutinating
3. Frequently necrotizing	3. Rarely necrotizing
4. Highly toxic to mice	4. Weakly toxic to mice

Within O-groups 4 and 6 we have exclusively to do with L-antigens (thermolabile envelope antigens) in so far as these types possess K-antigens at all, whereas in O-groups 8 and 9 we have partly A and partly L or B antigens. Within O-group 9 the A-antigens, i. e. thermostable capsule antigens, which are different from L-antigens, are greatly predominant, however.

These investigations provide no information as to the active principle that causes haemagglutination.

Conclusion.

The distribution of haemagglutinating Coli strains among the various serotypes and O-groups is described.

dilution is distributed on a series of test tubes, 10 cc. in each(and dilute bentonite sol is added to the tubes in increasing amounts — as described under titration of fibrinogen (section »Bleeding«). After standing for 10 min., precipitation of fibrin appeared in tube No. 13, corresponding to an addition of 13 % bentonite sol to the serum dilution 1 + 3.

The main portion, 30650 cc., is mixed with 50 l. distilled water, in which 460 g. sodium acetate has been dissolved. The mixture is acidified to pH 5.5 with hydrochloric acid. Under vigorous stirring, the calculated amount of bentonite sol, 15940 cc., is added.

After standing for one hour the mixed precipitate is centrifuged.

The centrifugate is neutralized with ammonia to pH 7.0.

Filtration through Seitz EK. plates.

Salting-out of the filtrate (88 liters) in 55 % saturated ammonium sulphate by solution of 25.5 kg. autoclaved $(\text{NH}_4)_2\text{SO}_4$ in the fluid.

The salting-out product is separated by filtration, then pressed and dialyzed as described in the preceding sections.

The dialysate amounts to 8000 cc., with the protein content of 8.6 %, and an antitoxin titer of 850 A. U. per cc.

Antitoxins prepared with employment of this simple technique will still contain a greater or smaller amount of euglobulin, and hence it is not advisable to use these preparations in the human therapy.

Summary.

A detailed description is given of the preparation of purified antitoxins from the blood of immunized horses, besides of the apparatus and reagents used for the preparation.

The blood is defibrinated with bentonite. Blood corpuscles and fibrin are removed by centrifuging.

The solution of serum proteins is submitted to proteolysis with pepsin in acid fluid.

The unspecific protein is removed from the solution by adsorption with aluminium hydroxide gel.

The solution of antitoxin-carrying protein is concentrated by ultrafiltration, salting-out and dialysis.

The preparations thus obtained are of pure pseudoglobulin character.

The preparations are tolerated well by the patients treated with them. They keep, unchanged, and the solution remains clear, without any sediment, even on storage for years.

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SOME UNUSUAL MUCOID ORGANISMS

By Sverre Dick Henriksen.

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In a previous study of chronic respiratory diseases (1), the observation was made that it was not unusual to isolate more than one mucoid organism from the same culture. It was thought that this occurred more frequently than one would expect as a chance coincidence.

The present study was undertaken in order to seek more information as to the significance of this observation.

A number of different mucoid strains were isolated for further study. Among these, there were some unusual organisms, which are described below.

A. A mucoid strain of *Pseudomonas aeruginosa*.

A blood agar culture from a case of chronic bronchitis was found to contain a number of mucoid colonies, which looked like typical *Klebsiella* colonies. The only unusual thing about the culture was a strong smell like that of pyocyanus cultures, although no colonies of this species were found. When the culture was left on the desk for a couple of days, some of the mucoid colonies were found to dry out rapidly, leaving only a thin, shrivelled granulated film, while others remained moist and raised. On subculture, the latter colonies were found to represent the usual type of *Klebsiella*, found in such conditions, whereas the quick-drying type was found to produce a yellowish-green pigment, often a metallic lustre of the surface of the colonies, and the characteristic odor of *Pseudomonas aeruginosa*. The latter strain had the following properties:

Morphology: Short gram-negative rods like typical pyocyanus bacilli. Stained by the India ink method of Butt, Bonyng and Joyce (2), they were found to be surrounded by somewhat irregular capsular

spaces. There also seemed to be a considerable quantity of intercellular material, probably free capsular substance. The rods were non-motile.

Colonies: 24 hours old colonies are indistinguishable from *Klebsiella* colonies, raised, strongly mucoid, of a thin, syrupy consistency. After 48 hours at 37 C or 3—4 days on the desk the colonies shrink to thin granulated films, usually with a metallic lustre. (Photo no. 1).

Subcultures from young, mucoid colonies usually gave a pure growth of the same type of colony, whereas subculture from older colonies gave a mixture of mucoid and non-mucoid colonies. Thus the strain had a marked tendency to produce non-mucoid mutants, this tendency being most marked at 37 C, less at lower temperatures. The non-mucoid colonies were of two types, one just like the usual pyocyaneus colony, the other smaller, smooth, raised with a navel-shaped depression on the top. This latter type was unstable and changed into the former type on subculture.

By always selecting strongly mucoid colonies for subculture, it has been possible to maintain the strain in the mucoid state for about 2 years so far.

Cultural and biochemical properties: Good growth on all the usual media between room temperature and 37 C. In broth even turbidity and a surface pellicle, later a viscid sediment. Poor growth under a vaseline seal.

Potato: thick brownish growth with a metallic lustre.

A yellowish green pigment is produced on all media.

Fermentation: Weak acid production from glucose. Maltose, lactose sucrose and mannitol are not fermented.

Milk: Coagulated in 48 hours, later slowly peptonized.

Gelatin: Sacculiform, later complete, liquefaction.

Serum slants: No liquefaction.

Nitrates are reduced to nitrites, but indol is not produced in peptone broth.

A solution of dimethyl paraphenylene diamine gives a positive oxydase reaction.

H₂S is produced in lead acetate agar.

Classification: The strain must be considered as a mucoid strain of *P. aeruginosa*.

Comment. Mucoid strains of *P. aeruginosa* are rare in human material. They are also stated to be rare elsewhere in nature. Such strains have been described previously by Sonnenschein (3), by Dahr and Kolb (4) and possibly by Pottien (3). Reid, Harris, Naghski and Gatchell have studied the dissociation of this species and have seen the mucoid form (5). The original reports on these forms were not available.

The simultaneous presence of this strain and a typical strain of *Klebsiella* in the same sample should be noted.

B. A mucoid strain of *Staphylococcus aureus*.

A throat swab from a case of chronic rhino-pharyngitis yielded practically pure growth of a strongly mucoid organism with white or ivory colored colonies. It was found to consist of gram-positive micrococci.

Morphology: Gram-positive cocci of the size, shape and arrangement of staphylococci. Stained by the India ink method (2) they were found to be surrounded by distinct capsular spaces.

Colonies: Large, 3—4 mm after 24 hours, moderately raised, very moist and confluent, of a thin, not markedly viscid consistency. The pigment varied in different cultures from pure white to pale yellow, and usually was concentrated in the centre of the colony, whereas the periphery was colorless or greyish, transparent. The yellow color was most marked in cultures cultivated at room temperature. The strain has remained quite stable in the mucoid state for about 2 years, with no tendency to dissociate (Photo no. 2).

Cultural and biochemical properties: Good growth on all the usual media. Broth cultures showed even, dense turbidity.

Fermentation: Acid was produced from glucose, maltose, lactose, sucrose, mannitol, dulcitol, trehalose, levulose, dextrin and inulin, but not from salicin, aesculin, rhamnose, arabinose, xylose, sorbitol or starch.

Gelatin: Sacculiform liquefaction from the 3rd or 4th day.

Milk: coagulated within 14 days.

Nitrates are reduced to nitrites but neither indol nor H_2S are produced.

No distinct hemolysis on blood agar.

Crystal violet agar (1:300000): growth with violet colonies which later turned yellow in the centre.

0.017 % bromothymol blue agar: good growth.

Plasma coagulase test: positive in $1\frac{1}{2}$ hours.

Mice were killed by intraperitoneal injection of 0.5 ml 24 hour broth culture, but not by 0.05 ml.

Classification: This strain gives the reactions of the pathogenic type of *Staphylococcus aureus*: fermentation of mannitol, violet growth on crystal violet agar, growth on bromothymol blue agar, and plasma coagulase test. The production of pigment was weak and irregular, but sufficient to show that it must be the aureus variety.

Comment. Mucoid forms of *Staph. aureus* have been described by Sonnenschein (3), Oesterle (6) and by Gilbert (7). All strains produced a strong yellow pigment, and the two latter strains were pathogenic to mice.

Bigger, Boland and O'Meara (8) studied the dissociation of staphylococci and found some »viscid« forms, which, however, seemed to be more closely related to the R-form than to the M-form. Apparently they were not encapsulated.

Nothing seems to be known as to the cause of the mucoid transformation.

C. A mucoid diplobacillus and an unusual strain of Klebsiella.

A nose swab from a case of chronic rhino-pharyngitis, among other organisms, gave growth of a pneumococcus type III and some fairly large, dry colonies with a navel-shaped depression. After the culture had been left on the desk for some days, an additional type of colony had appeared. This was a strongly mucoid colony of the *Klebsiella* type.

Subculture at 37 C in an electric incubator gave extremely poor, dry growth of both the navel-shaped colony and the mucoid one. At 30 C, however, both colonies gave a luxuriant, strongly mucoid growth, indistinguishable from that of *Klebsiella*. Further study showed that the navel-shaped type of colony conformed with *Klebsiella* in all respects except its unwillingness to grow at 37 C under usual conditions, whereas the mucoid colony from the primary culture was found to be a diplobacillus, the properties of which are reported below.

Both these strains were peculiar in their demand for a humid atmosphere. When cultivated at 37 C in the incubator, the *Klebsiella* only produced a very meager, dry growth on the part of the plate where the inoculum had been deposited, and the diplobacillus either failed to grow altogether, or produced an extremely meager, dry growth. When films were prepared from such growth, both strains showed extreme pleomorphism, with large swollen rods and pale »ghost cells«. Large »spherical bodies« were also produced, and other bizarre forms. Dumbbell shaped cells were frequent in the diplobacillus. (Photos nos. 3—9).

When the same strains were cultivated at 37 C in a closed jar, containing water, they always gave luxuriant, strongly mucoid growth, which showed no pleomorphism on microscopical examination. Similar growth was obtained in an incubator at 30 C or on the desk. The pleomorphism could be reproduced at will by cultivating at 37 C in a dry atmosphere. (Photos nos. 3—9).

None of the other mucoid organisms studied have shown this extreme sensitiveness to drying, nor similar pleomorphism.

Apart from this peculiarity, the strain of *Klebsiella* behaved as most other strains, and there is no need to describe its properties in detail.

The mucoid diplobacillus had the following properties:

Morphology: When grown in a humid atmosphere, the strain produced short, plump rods with a marked tendency to appear in pairs or in short chains. Grown at 37 C in a dry atmosphere it showed the pleomorphism described above.

The rods were non-motile, and were surrounded by large capsular spaces in India ink preparations. In the pleomorphic organisms the capsules still seemed to be present but very much reduced in size.

Cultural and biochemical properties: Luxuriant, mucoid growth on all solid media, if a humid atmosphere was provided. Very poor or no growth on all media in a dry atmosphere at 37 C. The colonies, when drying was prevented, were large, raised, confluent, moist, moderately viscid, just like most strains of *Klebsiella*.

In liquid media growth was very poor at 37 C, better at 30 C, both in plain and rich media.

No carbohydrates were fermented, neither indol nor H_2S produced and neither gelatin nor serum liquefied.

Nitrates were reduced to nitrites, and a positive oxydase reaction was obtained with dimethyl paraphenylenediamine.

Mice were killed by intraperitoneal injection of 500 million and 50 million organisms, but not by smaller doses.

Classification: This strain does not conform with any species listed in Bergey's manual, nor with any described in the available literature. In a recent study (9), however, some diplo-bacilli from the genito-urinary tract were described, which show a marked similarity with this strain. Such strains were also found in the respiratory tract. These diplobacilli were not mucoid, but otherwise had all the properties of this mucoid strain. They were thought to be related to *H. duplex*, and to represent a new species of diplobacilli.

It seems that the mucoid strain described above must be the mucoid form of this species, the classification of which has been discussed previously (9).

Comment. This is another case where two different mucoid gram-negative rods were isolated from the same case of chronic inflammation of a mucous membrane. Both strains grew with exactly the same type of mucoid colony, showed the same sensitiveness to drying, and a similar pleomorphism, when exposed to a dry atmosphere at 37 C. Such behaviour is unusual in *Klebsiella*. None of the other strains of *Klebsiella* studied were sensitive to drying. In the diplobacillus, on the other hand, it may be the normal behaviour. This type of diplobacillus has been shown to be unwilling to grow in a dry atmosphere at 37 C. But none of the strains, described previously, were mucoid. Thus it may seem as if these two strains, growing together on the same membrane, had borrowed some characteristics from each other.

It may be wise, however, to consider this just a set of unusual coincidences, in the absence of more tangible evidence.

It is interesting to note that the mucoid diplobacillus was pathogenic to mice, whereas the non-mucoid strains studied previously, were non-pathogenic even in large doses.

Summary.

Some organisms, rarely seen in the mucoid stage, are described. One was a mucoid strain of *Pseudomonas aeruginosa*, found together with a strain of *Klebsiella*.

The second was a mucoid *Staphylococcus aureus*.

The third and fourth strains, isolated from the same case, were a strain of *Klebsiella* and a mucoid diplobacillus. The colonies of these strains were indistinguishable from each other, and they both were very sensitive to a dry atmosphere at 37 C. They showed a marked pleomorphism, characterized by large, swollen cells and spherical bodies, when incubated at 37 C in a dry atmosphere.

The mucoid diplobacillus was pathogenic to mice, whereas the smooth stage of the same organism has been found to be non-pathogenic.

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Photo no. 1.
Mucoid strain of *P.*
aeruginosa $\times 5$.



Photo no. 2.
Mucoid *Staphylococcus*
aureus. $\times 4$.



Photo no. 3.
Klebsiella, incubated in
moist atmosphere.
 $\times 4$.



Photo no. 4.
Diplobacillus, incubated
in moist atmosphere.
 $\times 4$.

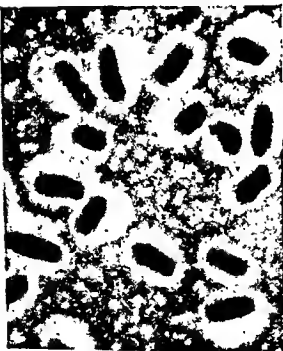


Photo no. 5.
Klebsiella, humid ath-
mosphere. India ink
preparation. $\times 1850$.

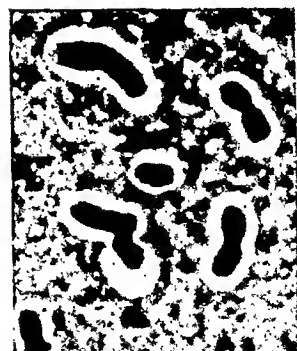


Photo no. 6.
Diplobacillus, humid
atmosphere. India ink
preparation. $\times 1850$.

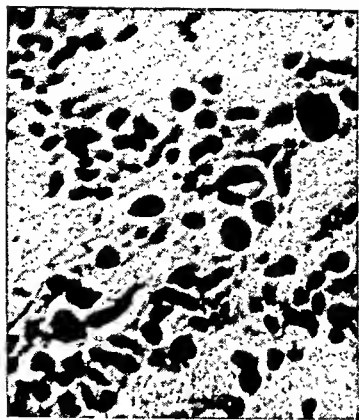


Photo no. 7.

Klebsiella. Dry atmosphere at 37 C. Pleomorphic organisms with large swollen cells. Gram's stain. $\times 1100$.



Photo no. 8.

Diplobacillus. Dry atmosphere. Pleomorphic organisms. Gram's stain. $\times 1850$.

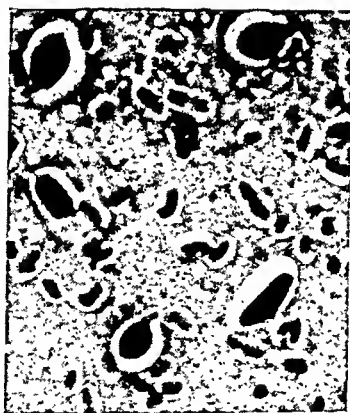


Photo no. 9.

Klebsiella. Dry atmosphere at 37 C. India ink preparation. Pleomorphic organisms with irregular capsules.
 $\times 1850$.

SEROLOGICAL CROSS-REACTIONS BETWEEN UNRELATED MUCOID ORGANISMS

By *Sverre Dick Henriksen.*

(Received for publication November 28th, 1947).

In previous papers (1,2) it has been shown that two or more mucoid organisms can sometimes be isolated from the same patient. This seems to be true particularly in certain chronic diseases of the mucous membranes, which practically constantly give growth of microbes classified as *Klebsiella*.

Several explanations might be suggested to account for this fact. 1. To start with the simplest explanation, it might be a mere chance coincidence. Study of a large material, collected from such conditions and from normal persons, would be necessary to prove or disprove this hypothesis.

2. It might be due to some selective factor, favoring the establishment of mucoid organisms on the mucous membranes, e. g. some agent inhibiting the growth of certain organisms in the smooth, but not in the mucoid state. The predominance of *Klebsiella* strains in such conditions could be explained by assuming a higher mutation rate in the bacteria which give rise to the *Klebsiella* strains (possibly *Escherichia*), than in other organisms. Only bacteria with a comparatively high mutation rate might have a reasonable chance of establishing themselves in the mucoid state, before being suppressed by the selective agent.

3. The studies of Avery et al. (3,4,5) and of Boivin et al. (6,7) on type transformation suggest the possibility of transfer of gene material between two organisms growing in the same place. So far, type transformation has only been observed within the same species. Whether it is possible between more distantly related or unrelated species is unknown.

These three hypotheses would not necessarily exclude each other.

An antigenic study of mucoid organisms, isolated from the same patient, might possibly give some indication as to which hypothesis was most likely in a given case.

Material and methods.

The strains studied include the unusual strains described in the preceding paper (2), namely:

A mucoid strain of <i>Pseudomonas aeruginosa</i> ,	— Strain P—752
A <i>Klebsiella</i> strains for the same patient,	— Strain K—752
A mucoid diplobacillus,	— Strain D—H4
A <i>Klebsiella</i> strain from the same patient,	— Strain K—H4
A mucoid <i>Staphylococcus aureus</i> ,	— Strain LKA

In addition, 25 other mucoid gram-negative rods, collected from various sources, were included in the study. These strains were isolated from the following sources:

Chronic rhino-pharyngitis (incl. ozaena)	7 strains
Asthma and chronic bronchitis	8 strains
Bronchopneumonia	2 strains
Pleurisy	1 strain
Liver abscess	1 strain
Purulent infections	2 strains
Urinary infections	3 strains

The cultural and biochemical reactions of these strains were studied in some detail, in order to get an idea as to which types were prevalent.

Serological methods.

Immunization: Rabbits were immunized by intravenous injections of formalin-killed bacterial suspensions on the first 5 days of each week for 3—4 weeks. Dosage started about 500 million organisms and increased gradually up to about 5000 million. Additional courses were given if test bleedings were found to be of insufficient titer. **Serological tests:** Attempts to utilize the agglutination reaction were not successful. Satisfactory titers were not obtained. The complement fixation reaction gave more promising results, but considerable work was necessary to find the most satisfactory method. The first attempts were made with incubation in a water bath at 37 C for 30' to 1 hour. Whereas homologous reactions usually were satisfactory with an incubation time of 1 hour or even 45', some cross-reactions gave variable results, and after incubation for 30', even the homologous reactions became inconsistent. It appeared as if the reaction with *Klebsiella* antigens was somewhat sluggish, and required considerable time to be completed. This may possibly be due to the high viscosity of the antigens.

Incubation at 4 C for 20 hours gave more satisfactory and reproducible results. The technique finally adopted was the following: Two-fold dilutions of inactivated immune serum were made up in 0.2 ml volumes, starting with 0.1 ml of serum (the dilutions were actually prepared in larger volumes, to obtain greater accuracy, and 0.2 ml volumes pipetted into a series of tubes).

Antigen: 0.2 ml of saline suspensions of heat-killed organisms, standardized to contain between 1000 and 2000 million cells per ml. Complement: 0.2 ml volumes, containing 2 hemolytic units (the unit determined as the smallest quantity necessary to give complete hemolysis with 0.4 ml 1.5 % sheep blood corpuscles, sensitized with 4 units of hemolytic amboceptor).

Incubation at 4 C for 20 hours. After incubation, addition of 0.4 ml of sensitized sheep blood corpuscles, and renewed incubation at 37 C. The tests were read after 15', and again after complete sedimentation of the blood corpuscles at room temperature.

Titers are given as the serum dilution in the total reaction mixture of 0.6 ml, in the last tube that gives at least 50 % fixation (i. e. less than 50 % hemolysis). Thus the serum dose 0.1 ml would correspond to the titer 1:6.

Experimental.

A. The properties of the Klebsiella strains.

Type of colony: Two different types of colonies could be distinguished with comparative ease. The predominant type (21 strains) was large, moderately raised, confluent, of a thin, syrupy, not very viscid consistency. The second type (2 strains) was larger, domed, of a firmer, more viscid consistency, like a sticky jelly. 4 strains differed slightly from the others, the colonies, while resembling the predominant type, were smaller, and one of them showed a marked tendency to split out smooth mutants. 3 of these strains were isolated from urinary infections. These strains differed from the others in other respects also, and were considered as mucoid strains of *E. coli*, rather than *Klebsiella*, that is if there is any real difference between the two.

Fermentation reactions: On bromothymol blue lactose agar only 4 strains produced an acid reactions. 23 strains grew with blue colonies. All strains produced acid from glucose, maltose and mannitol. Lactose was fermented by all but 4 strains after 24 hours to 7 days, and sucrose was fermented by 4 strains only. Only 8 of the strains produced gas, 3 urinary strains produced indol, and 3 of the strains, two of which were urinary, were weakly motile. 4 strains produced H_2S .

All strains gave a positive methyl red test, and all, except one, a negative Voges Proskauer test.

Thus a similar variation was found in this material as in most previous investigations of this group.

B. Complement fixation reactions of *Klebsiella* strains.

Complement fixation tests were set up in two different anti-*Klebsiella* immune sera (serum anti-K-H4 and serum anti-K-752), with 25 different strains as antigens. The homologous titer of both sera was 1:192. 18 strains gave reaction up to the homologous titer, within the experimental error. All variations on repetition of the tests, were within one tube below or above the homologous titer. 7 strains gave negative reactions, including 3 strains from urinary infections, the two strains with the domed colony, 1 ozaena strain and 1 broncho-pneumonia strain.

Thus the majority of mucoid strains isolated from various chronic conditions of the mucous membranes seem to belong to the same sero-type.

Table 1.

Cross-reacting antibodies to mucoid organisms demonstrated by fixation of complement. Antibody titers with constant antigen dose.

Immune sera	Antigens				
	<i>Klebsiella</i> K-752	<i>Klebsiella</i> K-H4	<i>Diplobacillus</i> D-H4	<i>Staphylo-</i> <i>coccus</i> . LKA	<i>P. aeruginosa</i> P-752
Anti K-752	192	192	192	0 §	0
Anti K-H4	96	192	192	24 §	0
Anti D-H4	48	48	3072	96	—
Anti LKA	0	0	192	192	—
Anti P-752	0	0	—	—	768

— : reaction not carried out.

§ : These reactions gave variable results.

C. Cross-reactions between *Klebsiella* and other mucoid organisms.

Immune sera were prepared as described above against the mucoid strain of *P. aeruginosa* (anti-P-752), the mucoid diplobacillus (anti-D-H4) and the mucoid *Staphylococcus* (anti-LKA).

Complement fixation tests were set up with different antigens in these immune sera and the two anti-*Klebsiella* sera. All tests were repeated on several occasions, and the results found to be consistent. All variations seemed to be within the experimental error. The results are presented in table I.

The table shows that the diplobacillus cross-reacts in the two *Klebsiella* immune sera up to the homologous titer, whereas the *Klebsiella* antigens only show moderate cross-reactions in the anti-diplobacillus immune serum. Surprisingly enough, the mucoid *staphylococcus* shows marked antigenic similarity with the diplobacillus. The slight, and somewhat irregular, cross-reactions in anti-*Klebsiella* im-

mune sera with the staphylococcus antigen may indicate a slight antigenic similarity, but the reactions were not entirely convincing.

The strain of *P. aeruginosa* showed no cross-reactions with the *Klebsiella*, isolated from the same patient. This negative reaction was confirmed by tests with crude, but probably protein-free, capsular polysaccharides, prepared from the two strains. These polysaccharides gave homologous precipitation and complement fixation only.

The cross-reactions between the diplobacillus and the *Klebsiella* from the same patient are of particular interest in this study. The question arises whether these, strong, but not complete, cross-reactions would be consistent with the 3rd hypothesis, suggested above, i. e. whether the cross-reactions might possibly be due to identical capsular polysaccharides. As it is unknown to what extent the capsular and somatic antigens contribute to the reactions in these cases, more detailed analysis would be necessary to answer the question.

The cross-reaction between the diplobacillus and the mucoid staphylococcus, although interesting, was not thought to be equally pertinent to this study, and was left for future consideration.

D. Preparation and serological assay of capsular polysaccharides.

In view of the fact that the *Klebsiella*, strain K-H4, gave complete cross-reactions with a majority of *Klebsiella* strains, it was thought most likely that it was a representative of Julianelle's group A. The method used by Goebel and Avery (8) for the preparation of the capsular polysaccharide of this group was consequently chosen as the one most likely to succeed.

30 agar plates each were inoculated with the two strains. After 48 hours at 30 C (under which conditions a luxuriant mucoid growth was always obtained), the growth was harvested and suspended in 300 ml of water, after which the directions given in (8) were followed as closely as possible.

The yields of capsular polysaccharide were 0.45 G from strain K-H4 and 0.1115 G of strain D-H4. The yields were insufficient for a complete chemical analysis, but some properties were examined. Both polysaccharides were greyish-white amorphous substances, which were fairly easily soluble in water (more easily in dilute alkali). Watery solutions were slightly opalescent, very viscous and gave a rather high acidity. About 2 % solutions of the polysaccharides had a pH of 2.22 for K-H4 and 2.37 for D-H4. The biuret reaction was negative in fairly concentrated solution, and the Molisch test positive. One of the solutions reduced Fehling's solution (D-H4), the other only after acid hydrolysis. Some other properties are shown in table II. For comparison the properties of Friedlander polysaccharides, as stated in (8) and (9) are shown in the same table.

It seems obvious that the degree of purification in these experiments was less satisfactory than in the studies referred to above. It

Table 2.

Properties of polysaccharides K-H4 and D-H4, compared with Friedlander types A, B and C polysaccharides, according to (8) and (9).

Origin of polysaccharide	Acid equivalent	(α) D	Nitrogen	Highest dilution giving precipitate in immune serum
Type A	430—445	—100° to —105°	0	1:2×10 ⁵
Type B	670—722	+100° to +102,5°	0	1:2×10 ⁵
Type C	575—681	+90° to +101°	0	1:2×10 ⁵
Strain K-H4	604	+62°§	1.5%	1:2×10 ⁵ §
Strain D-H4	578	+10°§	2.6%	1:2×10 ⁵ §

§: measured at pH 7.

§: Slight trace of precipitate after centrifugation with this dilution.

is felt that the small quantity of nitrogen probably represents some impurity. Serological tests, however, indicated that the degree of purification might be sufficient for the purpose of this study.

It is reasonably certain, when comparing the optical rotations of the different polysaccharides, that the one prepared from strain K-H4 is different from that of Julianelle's group A, contrary to expectation. It may however be identical with that of group B or group C. Studies of the reactions of authentic type-strains will be necessary to decide which. At any rate the sero-type, found to predominate in this collection of strains, seems to be another one than in Julianelle's material (10).

The differences in the optical rotations of the polysaccharides from strain K-H4 (s-K-H4) and from strain D-H4 (s-D-H4) suggest that these polysaccharides are different, although the purity of the preparations may not allow definite conclusions.

Precipitation tests with various dilutions of the two polysaccharides in the two immune sera, gave homologous precipitation only. Each preparation reacted with homologous serum up to a dilution of 1:2 × 10⁵.

Complement fixation tests gave entirely different results, as shown in table III.

Both preparations produced complete fixation with the homologous serum up to dilution 1:3.2 × 10⁵, whereas cross-reactions did not go further than to dilution 1:3200 and 1:12800 respectively.

Thus the cross-reactions between the two organisms seem to be due, in part at least, to related capsular polysaccharides.

Some absorption experiments were carried out, in order to find out whether absorption of a serum with the homologous polysaccharide

Table 3.

Complement fixation with capsular polysaccharides. Constant dose of serum (0.04 ml) and four-fold dilutions of antigen.

Immune serum	Highest reactive antigen dilution	
	Antigen S-K-114	Antigen S-D-114
Anti K-114	$1:3.2 \times 10^6$	1:12800
Anti K-752	$1:3.2 \times 10^6$	1:12800
Anti D-114	1:3200	$1:3.2 \times 10^6$

ride would remove all of the cross-reactive antibody. The results, which were unsatisfactory, are presented in table IV.

Table 4.

Anticomplementary activity and specific combining power of immune sera, absorbed with capsular polysaccharides.

Immune serum Absorbed with		Anticomplementary titer	Specific combining power	
Anti K-H4	s-K-114 1:4000		Antigen K-114 1:192	Antigen D-114 1:384
	s-D-114 1:4000	1:96	1:192	1:3072
Anti D-H4	s-K-114 1:10000	1:24	1:384	1:384
	s-D-114 1:10000	1:24	1:48	1:3072

Absorption was carried out with equal volumes of serum and of the antigen dilution stated in the table. The bacterial antigens were standardized to contain 1000 million cells per ml.

In the first attempt, absorption was made with a quantity of polysaccharide, sufficient to give a slight antigen excess, as determined in precipitation tests. The absorbed sera became extremely anticomplementary, possibly indicating that the precipitation had been incomplete, and that small soluble antigen-antibody complexes remained in the supernatants. It was thought that this might have been due to a too large antigen excess, and a dose from the equivalence zone was chosen for the next attempt. After absorption with this quantity, allowing several days in the refrigerator for completion of the reaction, the supernatants were less anticomplementary than in the first experiment, but no reduction of the titers, either homologous or heterologous, could be detected.

Thus attempts to absorb the cross-reacting antibody were unsuc-

cessful. Thus it is possible that somatic antigens may have played a rôle in the cross-reactions, but it is perhaps more likely that the failure may be due, either to failure in selecting a proper dose for absorption, or to some degree of degradation of the polysaccharides in the process of purification, which has reduced their specific combining power. This would also account for the fact that cross-reactions with polysaccharides were weaker than one might have expected, after the strong reactions with the complete bacterial antigens.

Discussion.

It appears from the results that the predominant sero-type in this material, isolated mainly from mild chronic infections like ozaena and chronic bronchitis is different from the type found to predominate in Julianelle's material. It is possible that differences in the selection of the strains may account for this difference. Julianelle's strains to a larger extent originated in more severe infections, such as Friedlander pneumonia, a disease which seems to be rare in Norway. This question must be left open for future studies.

The complement fixation reaction seems to be well suited for the study of mucoid organisms, if sufficient incubation time is allowed for completion of the reaction. The reaction seems to be more sensitive when it is a question of detecting minor, cross-reacting antigenic components, than either the agglutination or precipitation reactions.

The failure of the precipitation technique to demonstrate the cross-reacting antigens in this study may possibly be due to the formation of small, soluble antigen-antibody complexes, which are too small to precipitate, but large enough to fix complement.

No decision could be achieved as to whether the cross-reaction between a *Klebsiella* and a mucoid diplobacillus from the same patient was due to cross-reacting capsular polysaccharides only. Ample evidence was obtained, however, to show that the two polysaccharides were not identical.

There is thus no evidence to indicate that the peculiar similarities between these two strains are anything more than coincidences. The results are further confirmation of the observation that cross-reactions between polysaccharides of different origin are fairly common.

The cross-reaction between a mucoid diplobacillus and a mucoid staphylococcus is of some interest, but a complete analysis of this reaction can not be offered at the present time.

Summary.

The predominating sero-type among a number of *Klebsiella* strains, isolated from various mild, chronic infections, appears to be some other type than Julianelle's type A.

A mucoid strain of *P. aeruginosa* was found to be serologically unrelated to a *Klebsiella*, isolated from the same patient.

A mucoid diplobacillus showed a marked cross-reaction with a *Klebsiella* from the same patient, and also with a mucoid *Staphylococcus aureus* from a different patient.

The former cross-reaction was found to be due, in part at least, to cross-reacting, but different capsular polysaccharides.

The complement-fixation seems to be more sensitive than other reactions in detecting antihodies against mucoid organisms of these types, and particularly, cross-reacting antibodies.

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ON HAEMAGGLUTINATION BY *ESCHERICHIA COLI*

By *F. Kauffmann*.

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Having worked on the serology of the Coli group during the past few years, especially on the distribution of the haemolytic strains among the various serotypes, it became natural to include the haemagglutination of the Coli bacteria in these investigations. Our previous experiments had made clear the fact that haemolytic Coli strains particularly often range among certain serotypes of the O-groups 4 and 6, whereas not one strain of O-groups 8 and 9 cause haemolysis.

As long ago as in 1902 R. Kraus and St. Ludwig reported on haemagglutination by bacteria. They demonstrated that a *Vibrio* strain (Paris) and a strain of *Staphylococcus aureus* haemolyticus agglutinated rabbit erythrocytes in test-tubes, whereas typhoid bacilli caused no agglutination. The authors gave no indication of whether Coli strains were tested in this respect.

Afterwards, in 1908, G. Guyot published further studies of bacterial haemagglutination and established the fact that many Coli strains possess the property of agglutinating the erythrocytes of various animals. He employed slide-agglutination and a 5 % suspension of erythrocytes in saline. He showed that formalin-killed bacteria retain their agglutinating property.

In 1943 L. Rosenthal wrote on the »Agglutinating properties of *Escherichia coli*«, and confirmed and amplified Guyot's results. For example, he demonstrated that heating for two minutes at 100° C. destroyed the agglutinability of the culture, whereas alcohol treatment left it unaffected. Of 70 Coli strains isolated from faeces and tested, 12 strains agglutinated human erythrocytes.

Own Investigations.

The tests were made with living Coli cultures from 20-hour agar plates. The culture was stirred direct with a needle into a drop of 10 % blood (in saline solution) on a slide. After the slide had been tilted back and forth for about thirty seconds the reaction was read with the naked eye; in doubtful cases a lens (8x) was used.

The blood used was from man, guinea-pig, horse, rabbit and chick.

Chick blood was chosen for the reason that experiments with viruses (Hirst test) have shown that this kind of blood is particularly suitable for haemagglutination. Some tentative tests showed that it is immaterial whether one employs 5 % or 10 % blood and whether the blood is washed or not.

In making the tests, each strain was tried against all five kinds of blood at once. A total of 112 Coli strains of known antigenic structure and known haemolytic property were tested. They represented the first 25 O-groups, with particular reference to O-groups 2, 4, 6, 8 and 9. Of the 112 strains, 34 gave no agglutination in the five kinds of blood tested, and 94 strains were not haemolytic.

Thus it was found, in complete accord with the results published in the literature, that Coli bacteria possessed of haemagglutinating properties are of frequent occurrence and that haemagglutination does not run parallel with the haemolytic property. Whereas almost all haemolytic strains (there was one exception) were haemagglutinating, many strains not producing haemolysis gave strong haemagglutination. In other words, the haemagglutinating property is more frequent than the haemolytic and is distributed — though not regularly — among all O-groups so far tested.

On this matter of regularity, the point is that O-groups 4 and 6 were outstanding, giving haemagglutination particularly often, whereas O-groups 8 and 9 possessed this property much less frequently. In the table will be found the results of tests with 14 strains of O-groups 4 and 6 and 14 strains of O-group 9. As twelve strains of O-group 8 behaved in the same manner as the strains of O-group 9, and eleven strains of O-group 2 as those of O-groups 4 and 6, I have omitted to show these results in tabular form.

The table shows that of 14 strains from O-groups 4 and 6, 12 caused haemagglutination, at least in one kind of blood but usually in several or all the samples tested. Of 14 strains from O-group 9, only 5 caused haemagglutination, and in most cases it was only weak. The strongest reactions were obtained with guinea-pig blood, followed by chick and horse blood, whereas rabbit and human blood agglutinated less strongly and less often. In other words, agglutination did not always occur in all five blood samples, human blood especially failing in some instances. Therefore, if it is the intention to test a large number of Coli strains for their haemagglutinating property, it will in future suffice to work with guinea-pig and human blood, the two extremes.

The difference between O-groups 4 + 6 on the one hand and O-group 9 on the other was most evident in the case of human blood, for 9 of 14 strains from the former group (4 + 6) gave a positive reaction, whereas only 2 out of 14 strains of the latter group (9) reacted positively. When chick blood was used, a total of 12 positive reactions was obtained in groups 4 + 6, but in group 9 only 4. Of 9

haemolytic strains in group 4 and 6, 8 were also haemagglutinating.

Whether or not the strains were possessed of L-antigen did not affect their haemagglutinating property, as both forms, L plus and L minus, of strain U 5/41 (O-group 1) agglutinated guinea-pig blood strongly, the other kinds of blood only weakly.

The haemagglutinating property was not destroyed by heating a bacterial suspension (in NaCl) at 70° C. for one hour in a waterbath, although this treatment killed the bacteria. But bacteria heated for 5 minutes at 100° C. no longer caused haemagglutination. Treating with 1 % formalin, though killing the bacteria, did not affect the haemagglutinating property.

Table 1.
Haemagglutination by Escherichia coli.

Strain	Antigen			Haemo- lysis	Erythrocytes from				
	O	K	H		Hen	Rabbit	Horse	Guinea-pig	Man
U 4/41	4	3 L	5	+	+	+	+	+	+
A 104a	4	3 L	.	+	—	—	—	—	—
Bi 7457/41	4	6 L	5	+	+	+	+	+	+
A 93a	4	12 L	5	+	+	(+)	(+)	+	+
Su 65/42	4	12 L	.	+	+	(+)	(+)	+	+
A 77b	4	52 L	4	—	+	+	+	+	+
A 103a	4	52 L	.	—	(+)	(+)	+	+	—
Bi 7458/41	6	2 L	1	+	+	+	+	+	+
Su 4344/41	6	13 L	1	+	+	+	+	+	+
PA 151	6	13 L	.	—	(+)	—	—	—	—
K 14b	6	.	10	+	+	+	+	+	(+)
F 8316/41	6	15 L	16	—	(+)	(+)	(+)	+	—
PA 236	6	53 L	.	+	+	+	+	+	+
A 12b	6	54 L	10	—	—	—	—	—	—
Bi 316/42	9	9 L	12	—	+	+	+	+	(+)
A 354b	9	26 A	10	—	—	—	—	—	—
Bi 449/42	9	26 A	.	—	—	—	—	—	—
K 14a	9	28 A	.	—	—	—	—	—	—
Bi 161/42	9	29 A	.	—	—	—	—	—	—
A 43c	9	30 A	19	—	—	—	(+)	(+)	—
A 292a	9	31 A	4	—	—	—	—	—	—
Su 3973/41	9	31 A	.	—	—	—	—	—	—
A 95b	9	32 A	5	—	+	+	+	+	(+)
H 36	9	32 A	10	—	+	(+)	(+)	(+)	—
A 45a	9	32 A	.	—	(+)	(+)	(+)	(+)	—
Ap. 189	9	33 A	.	—	—	—	—	—	—
A 55a	9	34 A	.	—	—	—	—	—	—
F 294a	9	36 L	.	—	—	—	—	—	—

Explanation of signs: + = positive, (+) = weak positive, — = negative.
Haemolysis = tested by horse erythrocytes.

Discussion.

The object of this investigation was solely to ascertain the distribution of the haemagglutinating Coli strains among the more important Coli O-groups and types, and therefore I refrained from fundamental examinations of the nature of haemagglutination (apart from the resistance test just referred to).

These results have helped to add one more indication to the already known differences between various O-groups and types. The special position occupied by O-groups 4 + 6 on the one hand, and O-groups 8 + 9 on the other, also becomes distinctly evident when haemagglutination is taken into consideration. Whereas haemolysis and haemagglutination are very frequent within O-groups 4 + 6, haemolysis is completely absent in O-groups 8 + 9 and haemagglutination is relatively rare. As moreover these two groups (4 + 6 and 8 + 9) according to Ewertsen and Sjöstedt's results also differ by the fact that the occurrence of toxic and necrotizing strains are much more frequent in the former group (4 + 6) than in the latter (8 + 9), the difference between them is definitely established.

I must specially point out that not all types within one O-group behave in the same manner, and therefore the O-group determination alone will not suffice to solve Coli problems; it must be supplemented with a type diagnosis.

On the basis of all results so far (results which I have recently gathered into a Coli-review) we may take it for granted that most types within O-groups 4 and 6 are possessed of higher pathogenicity than the types within O-groups 8 and 9. The main differences may be given schematically as follows:

Differences between types of the O-groups.

4 + 6	8 + 9
1. Frequently haemolytic	1. Non-haemolytic
2. Frequently haemagglutinating	2. Rarely haemagglutinating
3. Frequently necrotizing	3. Rarely necrotizing
4. Highly toxic to mice	4. Weakly toxic to mice

Within O-groups 4 and 6 we have exclusively to do with L-antigens (thermolabile envelope antigens) in so far as these types possess K-antigens at all, whereas in O-groups 8 and 9 we have partly A and partly L or B antigens. Within O-group 9 the A-antigens, i. e. thermostable capsule antigens, which are different from L-antigens, are greatly predominant, however.

These investigations provide no information as to the active principle that causes haemagglutination.

Conclusion.

The distribution of haemagglutinating Coli strains among the various serotypes and O-groups is described.

Haemagglutinating Coli strains occur more frequently among types of O-groups 4 and 6 than among types of O-groups 8 and 9. There is no direct connection between haemolysis and haemagglutination, and yet nearly all haemolytic strains at the same time are haemagglutinating. The number of haemagglutinating strains is much greater than the number of haemolytic.

Of all the types of blood tested (human, guinea-pig, horse, rabbit and chick), guinea-pig blood was agglutinated most strongly. One hour's heating at 70° C. did not destroy the haemagglutinating property of the Coli bacteria; this requires brief heating (5 minutes) at 100° C.

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FURTHER BIOCHEMICAL TESTS IN THE COLI GROUP

By F. Kauffmann and Beate Perch.

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In an earlier communication by Kauffmann & Perch: »Ueber die Coliflora des gesunden Menschen« as well as in a subsequent work by Kauffmann: »Ueber kulturelle Untersuchungen in der Coli-Gruppe« a report was made of the cultural behaviour of all the Coli strains we had analyzed serologically.

As these cultures were tested in the years 1941—42, we have now, after a period of five or six years, repeated some tests for the purpose of obtaining an idea of the constancy of the biochemical types. In addition, we have supplemented our earlier investigations, having tested all the strains with regard to nitrate reduction, Voges-Proskauer and methyl-red reaction, and their behaviour on Christensen's urea agar, the following media being employed for the reactions:

1) Nitrate Reduction:

Nitrite-free KNO_3	0.2 g.
Peptone	1.0 g.
aqua dest.	1000.0 c.c.

Tube in 5 c.c. amounts. Autoclave at 120°C . for 5 minutes 3 days in succession. Inoculate the medium and incubate 4 days at 37°C .

Solution A: Dissolve 8 g. sulphanilic acid in 1000 c.c. 5 normal acetic acid.

Solution B: Dissolve 5 g. alphanaphthylamin in 1000 c.c. 5 normal acetic acid.

Solutions A and B are mixed immediately before using and 0.1 c.c. of the mixture is added to each tube.

Positive reaction = red colouring within 10 minutes.

2) Voges-Proskauer and Methyl-red Reaction

K_2HPO_4 (Merck)	0.5 %
Witte peptone	0.5 %
Glucose	0.5 %
aqua dest.	

Mix K_2HPO_4 , peptone and distilled water, heat to boiling, filter, autoclave and add 0.5 % glucose. Tube in narrow tubes (about 1 cm. diameter) to a depth of 5 cm. Boil for 10 minutes. Inoculate and incubate 4 days at 37° C.

Divide the medium into two halves. To one half add about $\frac{1}{2}$ — $\frac{2}{3}$ volume of 20 % NaOH. Shake well and slope the tubes. An eosinlike fluorescence occurring within a couple of hours signifies the positive reaction. To accelerate the reaction a few grains of creatinum may be added. To the other half add 2—3 drops of a 0.25 % alcoholic methyl-red solution. Positive reaction is signified by scarlet, weak positive reaction by red-orange, and negative reaction by a yellow colour.

3) Urea Decomposition

Urea agar according to Christensen:

Glucose	1	g.
Bactopeptone	1	g.
NaCl	5	g.
KH_2PO_4	2	g.
Phenol-red	0.012	g.
Agar	20.0	g.
Aqua dest.	1000.0	c.c.

Bring the above ingredients to the boil. Adjust to pH 6.8. Tube in Wassermann tubes in 4 c.c. amounts. Autoclave. Add 0.5 c.c. of sterile (Seitz filtered) 20 % urea solution. Mix well and slope with a deep but and a short slope. Incubate overnight for sterility. Inoculate from a salt-solution suspension made from a 20 hours agar culture as used for Simmons' medium.

The result of the testing of 244 strains in the above media was that in the majority of cases the following could be noted:

Nitrate reduction:	positive
Voges-Proskauer:	negative
Methyl-red:	positive
Urea decomposition:	negative

In some instances the nitrate reduction was only weakly positive, and in two (strains H 515 b and A 121 a) the Voges-Proskauer reaction gave a weakly positive result. From this it appears that in almost every case the Coli cultures tested serologically by us were typical in their cultural behaviour.

In the repetition of the cultural investigations made five or six years earlier we tried the test strains for the first 25 O-groups and on the whole obtained the same results as then. As examples we give below merely the results of the first 10 strains:

- 1) Strain U 5/41 in the year 1941 fermented salicin after 3 days, but in 1947 after 4 days.
- 2) Strain U 9/41 in 1947 behaved exactly as in 1941. Dulcile was fermented on the 2nd day, salicin on the 3rd day.
- 3) Strain U 14/41 fermented rhamnose after 11 days in 1941 and

salicin after 2 days; in 1947 it fermented neither rhamnose nor salicin, even after 30 days' observation.

- 4) Strain U 4/41 behaved in the same way in 1941 and in 1947; dulcitol and salicin were fermented after 2 days.
- 5) Strain U 1/41 also behaved in the same manner at the repeated test.
- 6) Strain Bi 7458/41 fermented salicin after 2 days in 1941, in 1947 after 3 days, but otherwise behaved in the same way.
- 7) Strain Bi 7509/41 in 1941 fermented dulcitol after 3 days and rhamnose after 11 days. In 1947 it attacked dulcitol after 2 days and rhamnose after 6 days.
- 8) Strain G 3404/41 fermented salicin after 1 day in 1941, after 2 days in 1947.
- 9) Strain Bi 316/42 in 1947 behaved as in 1942 and attacked salicin after 3 days.
- 10) Strain Bi 8337/41 in 1941 fermented sucrose after 28 days, but in 1947 after only 4 days.

Finally it may be mentioned that the test strain of O-group 14, strain Sn 4411/41, in contrast to 1941 now slowly and weakly formed H_2S .

These examples, taken from the test strains for the first ten O-groups, should be sufficient to show that fermentation by the Coli types on the whole must be regarded as constant. Only in the case of certain media such as dulcitol, rhamnose, salicin and sucrose were there a few insignificant deviations in the repeated tests. As will be known, these irregularities chiefly affect those reactions which occur late, one strain for instance fermenting sucrose after 4 days, after 28 days, or not at all. We were unable to observe any change in respect of any of the other substances which were fermented promptly or not at all. For example, all strains had fermented lactose at once, and they behaved in exactly the same way at the repeated test. Only one test strain, which had been received from Vahlne (PA 236) in the interval, attacked lactose after three or four days.

As appears from Knipschildt's reports, among strains serologically belonging to the Coli group there are also those which do not attack lactose. Thus the failure to ferment lactose does not preclude the diagnosis of Coli.

In order to provide an idea of the results we have obtained with the media employed, we reproduce below the record of the tests with strain U 9/41, which is one of the commonest biochemical types:

Adonitol — ³⁰	Rhamnose + ¹
Dulcitol + ¹⁻²	Maltose + ¹
Sorbitol + ¹	Salicin + ³
Arabinose + ¹	Inositol — ³⁰
Xylose + ¹	Lactose + ¹

Sucrose — ³⁰	Simmons' glucose agar + ¹
Mannitol + ¹ gas	Simmons' citrate agar — ⁴
Glucose + ¹ gas	KNO ₃ + ⁴
Indole + ¹	Voges-Proskauer — ⁴
H ₂ S — ⁶⁰	Methyl-red + ⁴
Gelatine — ⁶⁰	Urea agar — ⁴

All 244 *Coli* cultures were tested in the above manner. We shall refrain from reproducing the complete fermentation formulae, however, since they will be published elsewhere. Nevertheless, in order to give an impression of the reaction in these media we show below the results with the strains of the first hundred O-groups. For simplification we designate a positive reaction after 24 hours with +, a delayed positive reaction with — +, and a negative reaction after 30 days with —. Thus, 11 +, 1 — +, 88 — mean that 11 strains were promptly positive, 1 strain fermented the medium late, and 88 strains gave a negative reaction. Failing gas formation, which was tested only in glucose and mannitol, was decided after 4 days' observation, as also the absence of growth on Simmons' agar. Failure to form indole was observed in 2 tubes tested after 1 and 2 days. The KNO₃, Voges-Proskauer, methyl-red and urea reactions were read after 4 days.

Cultural behaviour of 100 Coli strains.

Adonitol: 11 +, 1 — +, 88 —
Dulcitol: 40 +, 28 — +, 32 —
Sorbitol: 96 +, 3 — +, 1 —
Arabinose: 100 +
Xylose: 94 +, 5 — +, 1 —
Rhamnose: 85 +, 11 — +, 4 —
Maltose: 100 +
Salicin: 12 +, 67 — +, 21 —
Inositol: 1 +, 2 — +, 97 —
Lactose: 100 +
Sucrose: 22 +, 15 — +, 63 —
Mannitol: 100 +
Glucose: 100 +
Gas production (in mannitol and glucose): 99 +, 1 —
Indole: 92 +, 8 —
H ₂ S: 4 +, 96 —
Gelatine: 100 —
Simmons' glucose agar: 100 +
Simmons' citrate agar: 3 +, 97 —
KNO ₃ : 100 +
Voges-Proskauer: 100 —
Methyl-red: 100 +
Urea agar: 100 —

Considering the material of 244 strains as a whole, we shall single out only certain special points.

Adonitol was fermented by 29 strains, of which 7, all belonging to O-group 21, were late in acting. Only 1 strain (H 520 a) of O-group 21, which was not possessed of the complete O-antigen of this group, did not attack adonitol. Two strains were not gas-producing (E 14 a and A 203 a); 12 strains did not form indole and belonged to two different biochemical types: 6 of them did not grow on Simmons' citrate agar and did not produce H_2S , whereas 6 others grew on Simmons' citrate agar and produced H_2S .

It has already been mentioned that two strains gave a weakly positive Voges-Proskauer reaction. Two others (A 43 c and A 95 b) were »ammonia-weak«, i. e. they failed to grow on Simmons' glucose and citrate agar.

In particular we shall point out that we found 6 strains that grew on Simmons' citrate agar; they all produced H_2S but not indole. Three of them (P 6 b, P 6 c and P 11 c) had already been tested in 1941, when the positive reaction on Simmons' citrate agar had been observed in all three cases. Unfortunately, in our earlier communication »Ueber die Coliflora des gesunden Menschen« we neglected to mention this positive citrate reaction, with the result that in that and all subsequent works we wrote that we had worked only with citrate-negative Coli strains.

Since then, Vahlne has reported on a number of citrate-positive strains belonging serologically to the Coli group. He demonstrated that a strain termed *B. lactis aerogenes* by Escherich belongs to O-group 9.

The six citrate-positive strains, which we have now tested repeatedly, belong serologically to the following Coli O-groups.

P 6 b	to O-group	67
P 6 c	»	17
P 11 c	»	72
H 310 a	»	94
A 231 a	»	8
G 8	»	8

It appears from these results that strains capable of growing on Simmons' citrate agar can also be inserted into our diagnostic antigenic schema.

In conclusion, the point should be emphasized that the serological Coli types have kept constant through years of cultivating in the laboratory.

In other words, in this respect the Coli types set up behave in exactly the same way as the serological *Salmonella* types, whose constancy has also been proved.

Summary.

The authors report on further biochemical tests in the Coli group with regard to the nitrate, Voges-Proskauer, methyl-red and urea reactions. Moreover, after five or six years they repeated some other tests which on the whole demonstrated the constancy of the biochemical types earlier observed. Certain special types, *e.g.* such as grow on Simmons' citrate agar, are underlined.

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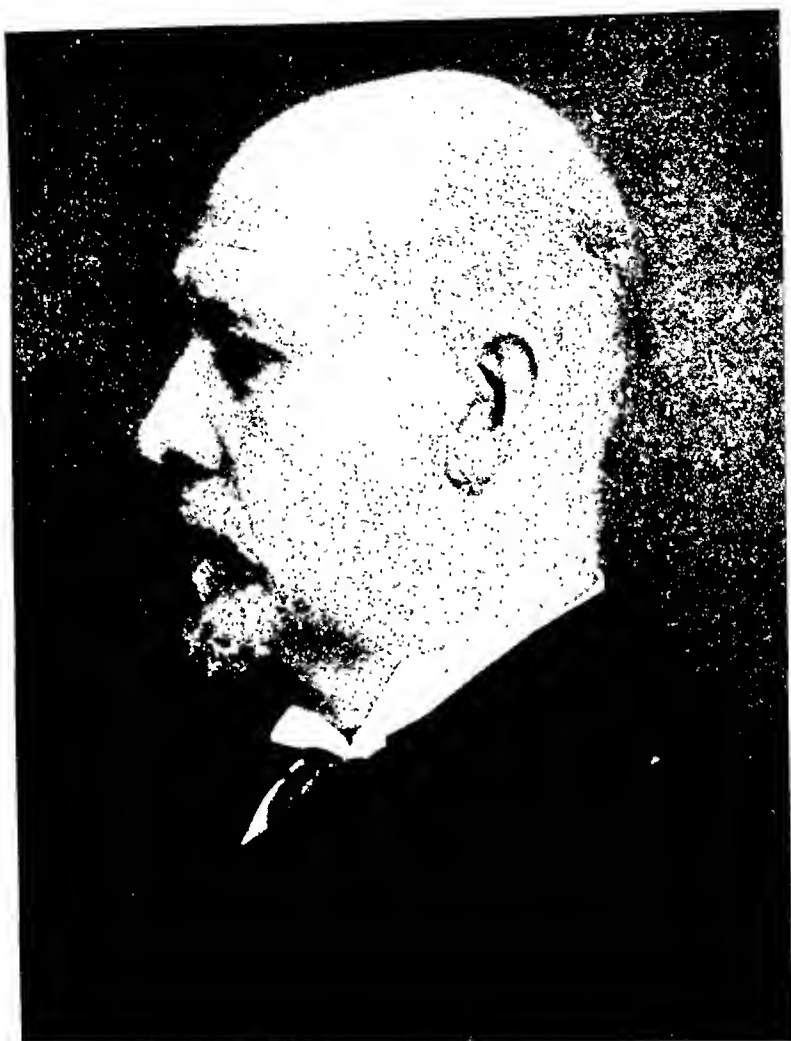
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JOHN FORSSMAN
22. 11. 1868 — 12. 3. 1947.



John Forssman

John Forssman was born at Kalmar and of well-known stock, renowned for its vitality and longevity. After matriculating at his native town in 1887 he immediately entered the University of Lund, where he took all his medical examinations which he finally crowned with a dissertation for which the degree of Doctor of Medicine was conferred upon him in 1898. As assistant at the Pathological Dept. of the University of Lund, Forssman received a thorough training in the fundamentals of pathology under the direction of the learned Professor M. V. Odenius, and it was from the prominent Danish bacteriologist Prof. C. J. Salomonsen, founder of the famous Serum State Institute in Copenhagen, and not least through a six months sojourn at the Pasteur Institute in Paris after his disputation that Forssman acquired a deep and wide knowledge of bacteriology. The young Swedish researcher soon won the affection of his teachers — especially that of Roux — and sowed the seeds of a lifelong friendship with several other bacteriologists of his own age. It was in Paris that Forssman began an extensive study of the bacteriology of botulism, which investigation he terminated on his return to Lund, where he tackled especially the problems connected with immunization.

Already Forssman's dissertation on the factors determining the direction of growth of cut nerve fibers during regeneration had aroused great interest and had been rewarded with a very high award. It was the first study in experimental pathology that had been carried out at Lund. In 1901 at the age of 32 and after publishing a few smaller studies, Forssman was elected Professor of General Pathology and Bacteriology, which chair he was to occupy for over thirty years. As to his teaching, there is but one opinion: he was a stimulating, captivating instructor who was capable of fusing life into his lectures in a marvellous way, often amusing but nevertheless at the same time commanding respect.

When we think of John Forssman, it is always as the researcher that he appears before us. Experimental investigation in the sphere of fundamental research was his guiding star through life; it was the very breath of his life without which he simply could not exist. But in his research work he never pursued trodden paths but always the unplotted course. This clear-sighted worker frequently could accept theories and dogmas with his constructive criticism; as a matter of fact it seemed as if he found pleasure in detecting deviations from established conceptions for which he then endeavoured to find better explanations. His most salient work in this respect consists of what has now become the classical works on so-called heterogenetic antibodies, which to-day bear his name and which constitute one of the most complicated problems of serology.

It is necessary to give a brief description of the situation shortly before this discovery. The keystone of bacteriology and serology is and always will be the law of specificity. Thanks to this recognition

we were able to extricate ourselves from the chaos that bacteriology formerly spelt, and the strict logic in Pasteur's and Koch's research at once became obvious to all. Thus, when Behring produced his anti-toxic diphtheria serum, it was a triumph for the specific-bacteriological treatment. An elucidation of the nature of the specific process was wanting, and Paul Ehrlich responded to this call with his famous side-chain theory, which was both genial in its simplicity, and more than useful as a hypothesis. But when the side-chain theory was dubbed an established fact by some of the enthusiastic pupils of Ehrlich (for example, Wassermann), it did not promote but instead thereof fettered the development of serology. Criticism was brought forward especially from the Lund researchers, John Forssman and Ivar Bang, who produced evidence that seemed to shake the very fundamentals of Ehrlich's hypothesis. By immunizing animals with blood cells Bang and Forssman had been able to produce antihodies capable of dissolving the blood cells, hæmolysins, and found that this antibody formation was dependent on the lipoid substances of the blood cells. It was also discovered that binding and antigenic properties are not always co-existent and that even the strict laws of specificity exhibit exceptions. During the subsequent scientific polemic against Ehrlich, Forssman sought systematically for deviations from specificity. From immunizing with cells, red blood cells, he switched over to the injection of emulsions of organs, especially those of guinea-pig kidneys, the choice of this organ probably being influenced by the dissertation by Gunnar Forssner published shortly before on the elective renal localization of bacteria cultivated on media containing kidney extract. It was with such extracts that Forssman immunized rabbits from which he then obtained hæmolysins against sheep blood cells, a result just as unexpected as it was remarkable. This discovery gradually prompted a lively research *inter alia* by Doerr, Sachs and Landsteiner concerning specific and non-specific factors of antigens. In 1928 in *Handbuch der pathogenen Mikroorganismen* Forssman gave a summary of his own studies and those of others in this field.

Even though Forssman's discovery did not provide a solution to certain baffling serological problems, the recognition of the heterogenetic antigens and their specific antibodies did at any rate suggest a plausible path to follow not only in bacteriology, but also in medicine as a whole.

It would be going too far to expatiate on all of Forssman's scientific products, but mention should be made of his thorough study of anaphylactic shock, the underlying chemical factors of Wassermann's reaction, non-specific vaccinotherapy, etc. For investigating the nature of so-called reverse anaphylactic shock Forssman's heterogenetic antisera have played an important role, and the assumption that the cellular changes are the essential factors, and the blood changes, secondary, was confirmed. By intracarotid-centripetal injection of

heterogenetic sheep-hæmolytic rabbit serum into guinea-pigs Forssman obtained a characteristic reaction known under the name of Forssman's intracarotid syndrome. He also showed that this phenomenon differs from anaphylactic shock. From his emeritus years there exist inter alia a series of reports on staphylococcal immunity. On account of the manner in which Forssman published these studies the results are hard to survey. Sometimes they are contradictory and not infrequently in disagreement with those of earlier and contemporary investigators. However, Forssman contends that explanations brought forward so far hardly hold good but must presuppose other hitherto unknown immunity factors.

At the 250th anniversary of the University of Lund, Forssman published a remarkable article in the University Annual. It was entitled »The dependence of pathogenic bacteria, infections and certain epidemics on foreign bacteria«. In this publication Forssman had accumulated reports on experiences made in various parts of the globe as to how epidemics can persist or die out, and in his usual clear-sighted manner showed how the survival or disappearance of diseases is dependent upon antagonistic factors existent in the soil. The problem is most distinctly depicted in his own words »Even if pure cultures did play an enormous role in the development of bacteriology, and even if it does and most probably always will, so to say, constitute the backbone of all bacteriological studies, it should nevertheless be borne in mind that in Nature bacteria seldom appear in pure cultures. In Nature we often encounter them in both quantitatively and qualitatively varying mixtures or, if you like, mixed cultures (for we imagine them in a state of development). If, for example, we look at the conditions ruling in the soil, in the water, in the mouth of man or animal, and in other parts of the digestive canal, etc., everywhere do we meet a mass of different bacterial species in varying proportions. And if we want to learn the role bacteria play in Nature, we must, after studying them in pure cultures return to work with mixed cultures because the total result of the activity of the mixed cultures of bacteria is by no means simply the sum of the activity of the individual species. On the contrary, the result will be totally different and will be dependent on the influence — sometimes stimulating, sometimes inhibitory — which the various species exercise on one another«. These prophetic words of Forssman are worth remembering, now in the era of antibiotica with penicillin, streptomycin, etc., and when soil bacteria have suddenly come into the focus of medical observation.

Forssman's love for scientific research had a sedative effect on his desire to be engaged for any length of time in public work. When such tasks were placed upon him he fulfilled them both quickly and effectively and returned to his research work. His laboratory at the old Dept. was his favourite place. If one happened to go there on Xmas day or New Year's Eve, one often met a lonely man at work at his

desk: the Chief. His dexterity was admirable, he was a skilful animal operator and to watch him blow glass was a pleasure which he himself obviously enjoyed. For anybody who has studied Forssman's experimental work from close quarters, it is evident that his desire to tackle the most baffling questions often induced him to grapple with problems which not infrequently refused to be solved, with the result that laboratory work of several years sometimes remained unpublished.

In spite of this, John Forssman was by no means a narrow-minded researcher. For many years he practiced as a dermatologist and venereologist and was Head of the Venereological Dept. His keen sense of responsibility and pronounced public spirit resulted in his being appointed Town Councillor, Director of Lund's Hospital, Pro-rector of the University, etc. In the Medical Society of Lund he was for many years one of the leaders. In a number of epidemic questions important for the welfare of his country, he gave many valuable contributions in such questions as small-pox inoculation, the combat of foot and mouth disease, etc. His sound practical judgement was esteemed by members of all classes and the public welfare was always foremost in his mind, personal aims or regards being unknown to him. His juvenile spirit gave him a deep understanding for the students at the University, as many traces at Lund bear witness to. He liked to see the youths merry and lively, and had no time for bores. His sound judgement was: If they are bores at this young age, what will they be when they get old? For good, healthy sinners he had a secret liking, sometimes not so very secret. He never sought popularity, and that is perhaps one of the reasons why he was liked so much and by so many. Great was the number of people who sought his wise advice, and they always discovered that they had chosen the right man.

Forssman's manly, blunt character reflected a lover of nature, fond of country life. Spartan in his daily life and in full possession of his physical and psychic powers he brought forward valuable and fascinating suggestions almost to the end of his days. He was granted a long rich life and was able to contribute admirably to his institute, his faculty, his university, his town and his country. He won international praise, and when he left us it was after a short illness without regression.

During his long life honours were granted him in abundance. He was a member of the Royal Academy of Science, Sweden, and of the Royal Academy of Science, Denmark, and in 1922 he won the Jubilee Prize of the Swedish Medical Society, and in 1924, the Björkén Prize of the Medical Faculty of the University of Upsala. When he left the chair in 1933 his colleagues and pupils honoured him with a memorial volume containing inter alia a bibliography of his own literary productions up to 1933.

He was a member of the Royal Physiographic Society of Lund

throughout 45 years during which he was very active and exhibited a more than warm interest. He was on the Advisory Financial Committee and the Editorial Staff of the Annual Publication. A large donation from John Forssman and his wife in 1928 bears witness to the interest he displayed in the Society. In 1927 he was granted the Gold Medal of the Royal Physiographic Society, and in 1940 was elected a member of honour.

The medical world, both at home and abroad, will remember him with gratitude and veneration.

Arvid Lindau.

EFFECT OF PENICILLIN ON PROTEUS VULGARIS

By *O. Lahelle*.

(Received for publication December 23rd 1947.)

As has earlier been pointed out, it is chiefly to the effects of penicillin on the sensitive microbes, i. e., the Gram-positive bacteria, that attention has hitherto been directed. We know, however, that also Gram-negative cocci may be very sensitive to penicillin. This applies, for instance, to *Neisseria gonorrhoeae* and *Neisseria intracellularis meningitidis*. Far less attention has been devoted to microbes which are to-day regarded as resistant to penicillin. Also in this field, however, some publications have appeared, in which it is mostly the Gram-negative intestinal bacteria that have been subjected to investigation. In a quite recently published paper *O. Lahelle* (1948) has dealt with some of these matters. Here it was shown that *Proteus vulgaris*, *Proteus morgani*, *Escherichia coli* and the coliform bacteria were sensitive to penicillin. At the same time, however, it was found that the penicillin-sensitivity varied considerably in these groups of microbes. Thus *Proteus morgani* was highly resistant to the antibioticum, while *Proteus vulgaris* showed relatively little resistance. *Escherichia coli* and the coliform bacteria occupied an intermediate position.

Moreover, also other authors have shown that *Proteus vulgaris* is comparatively little resistant to penicillin. *G. T. Stewart* (1945) and *A. R. Thomas* and *Max Levine* (1945) have had the same experience. *G. T. Stewart* tried local application of penicillin in cases of circumscribed infection due to *Proteus vulgaris*, and apparently with success. His material, however, was small and his results can hardly be said to justify general conclusions. Like *A. R. Thomas* and *Max Levine*, he maintains that in case of infections of the urinary tract with *Proteus vulgaris* there is every possible reason for trying penicillin treatment.

Author's investigations.

In these experiments 10 strains of the *Proteus vulgaris* were tested. One strain had a resistance corresponding to 63 units of penicillin per

ml. Two strains had a resistance of 32 units and the other strains had a power of resistance lying between 16 and 8 units per ml. The procedure adopted for measurement of the penicillin-sensitivity has been described in the above-mentioned work by the present author. The resistance accordingly varied somewhat in the different strains, but they were all relatively sensitive to penicillin. Thus experiments carried out confirmed the results arrived at by *G. T. Stewart* (1945) and *A. R. Thomas and Max Levine* (1945). We also agree with the view expressed by these authors that there is good reason to try penicillin treatment in case of infections of the urinary tract occasioned by *Proteus vulgaris*.

In addition to testing the penicillin-resistance there was also carried out a more detailed investigation of the mode of action of penicillin on *Proteus vulgaris*, and thus made a more exact examination of the problem respecting bacteriostasis and bactericidal action. As early as in 1929 *A. Fleming* found that penicillin exercised a bactericidal effect on the microbes. He observed that not only were staphylococcal colonies which had grown on an agar-plate containing colonies of *Penicillium notatum* inhibited in their further growth, but there took place a lysis of the colonies already formed. Meanwhile, around about 1941, when the investigations respecting penicillin began in earnest, the general opinion was that it was chiefly a question of bacteriostasis. This view was based upon investigations made by *E. P. Abraham et al.* (1941). But soon followed publications which distinctly showed that there also occurred a bactericidal effect. Here may be mentioned investigations made by *G. L. Hobby, K. Meyer* and *E. Chaffee* (1942), *L. A. Rantz* and *W. M. M. Kirby* (1944), as well as by *E. Chain* and *E. S. Duthie* (1945). *K. Riewerts Eriksen* (1946) employed direct agar-microscopy. Penicillin was added to the agar, and the growth of the microbes was observed in the same field of vision the whole time. By this method he was able to show that both staphylococci and pneumococci underwent lysis through the action of penicillin. At the same time it was noted that in the weaker concentrations of penicillin the bacteria showed primary growth before they decreased in number and became dissolved. Likewise *A. Fleming* observed such primary growth of staphylococci in penicillin-broth, with subsequent lysis. This finding has also been mentioned by several other authors, including *L. A. Rantz* and *W. M. M. Kirby* (1944), *G. L. Hobby, K. Meyer* and *F. Chaffee* (1942). While *K. R. Eriksen* employed direct agar-microscopy, the other authors mentioned used either viability counts or turbidimetric methods. *Eriksen* holds that viability counts alone do not give any clear idea of the primary growth, since simultaneously therewith there takes place a lysis of the bacteria which may counterbalance or exceed the new growth thereof. He also believes that measurement of the primary growth by turbidimetric methods is not satisfactory, as the new growth may often be greater than is revealed by turbidimetry.

We have ourselves carried out several investigations for the purpose of detecting the primary growth by counting the number of living microbes. The method seemed to be very uncertain and gave varying results, so that sometimes the primary growth could be noted, while at other times no such increased growth was observed, even with use of the same inoculum and the same penicillin concentrations. We must therefore agree with the view that viability counts are hardly suitable for detection of the primary growth. We have not had an opportunity of making turbidimetric measurements.

In the experiments with *Protens vulgaris* direct agar-microscopy was employed. For this purpose a moist chamber was used. The microbes were inoculated on a small agar-film on a coverglass. The coverglass with the agar-penicillin culture was then mounted on a concave slide and by means of vaseline along the edge of the coverglass the chamber was made airtight and watertight. On incubating the culture at 37° C. and at the same time microscoping at definite intervals the development of the microbes under the influence of penicillin could be observed. It is to be noted that the microscope with the moist chamber was kept in the incubator during the whole experiment, so that the same field of vision was under observation all the time. A typical experiment with one of our *Protens vulgaris* strains shall be described.

The results will best be seen from inspection of the photographs on the next pages. The living microbes were photographed by means of a Leica camera. By employing oblique illumination and oil immersion lens with iris-diaphragm we were able to take very good pictures. The inoculation was made with 20-hour-old broth culture, diluted to a suitable degree. Fig. 1 shows the microbes before they had begun to grow. Fig. 2 shows the microbes after two hours' incubation. As is seen, they have grown somewhat in length. Fig. 3, taken after incubation for 2 hours and 45 minutes, shows quite distinctly a growth in the length of the individual cells. *On the other hand, no increase in the number of bacteria could be noted.* Fig. 4, which was taken after 3½ hours' incubation shows long, threadlike bacteria, still without any increase in the number of cells. This photograph also reveals another peculiar phenomenon, namely, the appearance of spindle-formed or spherical thickenings, the so-called »large bodies«, on the separate threads. This phenomenon is still more conspicuous after 6 hours, while the threads at this time become somewhat less distinct and the large bodies show impressions at several places and are not so regularly formed as before. After 9 hours most of the threads are more or less broken up and form a tangled mass. Likewise the large bodies are in many places distinctly disintegrated and have irregular contours. Some of them seem to be in a state of complete dissolution. After 24 hours incubation the whole assemblage of cells is entirely broken up. The threads have now for the most part disappeared, or else only frag-

ments remain. The large bodies are relatively better preserved. In another experiment this phenomenon was still more distinctly observable. Here we often saw only the large bodies, while the threadlike elements had vanished entirely.



Fig. 1.
After 1 hours' incubation.



Fig. 2.
After 2 hours' incubation.



Fig. 3.
After 23¼ hours' incubation.



Fig. 4.
After 31½ hours' incubation.



Fig. 5.
After 6 hours' incubation.



Fig. 6.
After 9 hours' incubation.



Fig. 7.
After 24 hours' incubation.

It is to be noted that agar containing 13 units of penicillin per ml (Merek) was used in this experiment. On use of weaker concentrations of penicillin, for example, 5 units per ml, there came growth of long threadlike cells, in most cases with formation of large bodies. But here no subsequent lysis of the bacteria took place. On ordinary agar without penicillin the *Proteus* strain employed gave growth of rod-shaped bacteria without tendency to formation of threads.

Different strains of the *Proteus vulgaris* may vary greatly in their morphology. It is to be emphasized that the strain we employed gave no growth of long threads or large bodies on ordinary penicillin-free agar. With stronger concentrations of penicillin in the agar, for instance, 50 units per ml., no threadlike elements or large bodies could be observed. The microbes were then merely dissolved during incubation, without any characteristic morphological changes being detected.

As already mentioned, *K. R. Eriksen* (1946) was able with certainty to detect a primary growth of staphylococci and pneumococci. This is clearly revealed in the photographs accompanying his publication. In a later work from the same year he claims to have made the same finding with respect to the *Bacillus anthracis* and a particular paracolon strain. On closer inspection of the accompanying photographs, however, it is seen that there is no increase in the number of microbes, but only a growth in the length or an increase in the breadth of the individual cells. Also in our experiments with *Proteus vulgaris* this was found to be the case. No certain increase in the number of cells could be noted on inspection of the same field of vision during the time the experiment lasted.

As regards the morphological changes, it is especially to be remarked that the formation of large bodies is not a property characteristic of penicillin alone. *O. Molkte* (1927) found the same phenomenon in cultures on 1 per cent and 2 per cent phenol-agar. He does not state, however, whether this was a regular occurrence. *L. Dienes* has in several works given a detailed account of the development of the large bodies in many species of bacteria. He states that addition of lithium, calcium or chromium salts to the media promotes the development of these bodies, but at the same time he remarks that only small proportion of the microbes undergo such morphological changes. *A. R. Thomas* and *Max Levine* (1945) observed a great many large bodies when working with Gram-negative intestinal rods in penicillin-media. *Erna Altire-Werber and coworkers* (1945) have made the same observation. The development of the large bodies, however, was not followed step by step during the incubation, as was done in our experiments. We assume, as does *L. Dienes*, that the large bodies represent variation forms of the bacteria. The same author also found that in coli strains these bodies contained nucleus-like chromatin granules. It is reasonable to suppose that the same is the case with the large bodies that appeared in *Proteus vulgaris* under the influence of penicillin.

For several species of microbes, such as staphylococci, streptococci and pneumococci, it was found that the resistance to penicillin increased when they were cultivated in media containing increasing quantities of the antibioticum. The same observation was made by *G. T. Stewart* (1945) as regards *Proteus vulgaris*. We have been able to confirm this finding. In our experiments ordinary bouillon was used, containing at first 4 units of penicillin per ml. After incubation for 24 hours culture

was made from this tube in broth containing 10 units, afterwards in broth containing 20 units and so on. Four strains of *Proteus vulgaris* were investigated in this manner. In another series of experiments cultures were made alternately in media with rising quantities of penicillin and in penicillin-free media. As regards *Proteus vulgaris* both methods gave good results, and it did not appear that one method had any advantages over the other. Abundant inoculations were made in all cases. One of the strains, P. 1860, which at first just barely showed growth in broth with 4 units of penicillin, increased its resistance so greatly that at the conclusion of the experiment it grew in a medium containing 3000 units per ml. The other three strains at the close of the experiment showed growth in media with 1000 units per ml. Thus P. 1860 increased its power of resistance so much that it was capable of growing in a medium containing 750 times the quantity of penicillin that in the beginning had just barely allowed growth. In case of all four strains we investigated the question whether the increased resistance was due to the strains having acquired the power of producing penicillinase. No production of penicillinase could, however, be noted in the resistant *Proteus* strains. A. Bondi and Catherine Dietz (1944) had previously established that *Proteus vulgaris* does not produce the penicillin-destroying enzyme, and we were able to confirm their findings.

The growth in the different media changed its character to some extent when the strains became resistant. Whereas under usual conditions they caused uniform turbidity in ordinary bouillon after 20 hours' incubation, it was now seen that two of the resistant strains produced a pellicle on the surface and only slight turbidity in the rest of the medium. Further it was found that the penicillin-treated strains on inoculation on 2 per cent agar plates had lost the power of swarming, or else swarmed more slowly than the non-treated strains.

Investigations of the increased resistance to penicillin were also made by means of *in vivo* experiments. One strain was employed for this purpose, namely P. 1860, and $\frac{1}{2}$ ml of a 6-hour culture thereof was injected into four mice. The mice were then given 2000 units of penicillin twice in 24 hours. Some of the mice survived the infection, while others succumbed. The mice that became moribund were killed, peritoneum was washed with saline solution and $\frac{1}{2}$ ml of the suspension thus obtained was injected intraperitoneally into other mice. The penicillin was injected subcutaneously-intramuscularly into the groin. On passage through 20 mice the strain increased its resistance so much that it grew in bouillon containing 125 units of penicillin per ml. The increase in resistance was thus comparatively small and the strain did not become more resistant on passage through a further 20 penicillin-treated mice. Also here it was sought to ascertain whether the penicillin-treated strain was capable of producing penicillinase, but with negative result.

It has earlier been found that species of bacteria which show increased resistance to penicillin behave in different ways when they are again transferred to penicillin-free media. Thus *E. W. Todd, G. S. Turner* and *L. G. W. Drew* (1945) and *K. R. Eriksen* (1946) observed that staphylococci rapidly lose their power of resistance and again become sensitive to penicillin when growing in penicillin-free media. This applied to staphylococci which had shown increased resistance in experiments *in vitro*. As regards hemolytic streptococci *K. R. Eriksen* (1946) found that the resistance remains unaltered on cultivation in penicillin-free serum bouillon. It must be mentioned that the reports in the literature on this point are often conflicting, and the results attained by the various authors are not easily comparable on account of the different technique adopted in the investigations.

Our four strains of *Proteus vulgaris* were transferred to penicillin-free media after the resistance in the experiments *in vitro* had increased as described. After 10 passages through bouillon we found the following figures for the resistance of the separate strains; P. 1860, which had increased its resistance so that it could grow in 3000 units of penicillin per ml, was now resistant only in media with up to 500 units per ml. Of the other three strains, which just barely showed growth in media with 1000 units, one, P. 2354, had unaltered resistance, while the other two strains had a resistance corresponding to 125 units per ml. After a further 10 passages through penicillin-free bouillon two of the strains were resistant in media with 125 units per ml and the other two strains in media containing 16 units pr. ml. It is to be remarked that all the strains had originally showed resistance to 8 units of penicillin per ml. Thus they all became considerably less resistant after growth in penicillin-free broth, but the power of resistance declined with varying rapidity in the individual strains. On conclusion of the experiment two strains had almost completely lost their increased power of resistance, while in the other two strains the resistance was somewhat higher than originally.

In several species of bacteria it has been found that the strains lose their virulence when the resistance to penicillin increases. For example, *John E. Blair and coworkers* (1946) found this to be the case with staphylococci which had shown increased resistance in experiments *in vitro*. Similar observations are made as regards the hemolytic streptococci. Investigations made by *G. Rake and coworkers* (1946) and by *K. R. Eriksen* (1946) show that these microbes lose their virulence when the resistance to penicillin has been found to increase during *in vitro* experiments. With respect to pneumococci the reports vary. Most often we find it stated that some strains lose their virulence, while others retain their virulence, even if the penicillin resistance increases.

One of our strains, namely, P 1860, which had been rendered very resistant to penicillin by *in vitro* experiments, was tested on mice. Fifteen mice were given intraperitoneal injections of $\frac{1}{2}$ ml of a 6-hour culture and at the same time fifteen other mice received similar in-

jections of the original strain. After 24 hours 12 of the mice into which the resistant *Proteus* bacteria had been injected were dead, while 14 of the mice in the control group had died. After an observation period of five days the same result was recorded. Thus it seems as if the resistant bacteria had retained their virulence.

In the same series of experiments 15 mice were given injections in the same manner as the control group, but received at the same time subcutaneous-intramuscular injections of penicillin in the thigh, 2000 units twice in the course of 24 hours. The first dose of penicillin was given immediately after the mice had been infected by the bacteria. After 12 hours' observation, *i. e.*, after injection of 2000 units of penicillin, 13 of the mice in this group were still living, while in the corresponding control group, which did not receive penicillin, only one mouse survived. After 24 hours 8 of the penicillin-treated mice were living, while 14 of those in the control group had died. Accordingly it would seem pretty certain that penicillin has a decided effect on *Proteus* infection in mice.

Summary.

1. On titration of the penicillin sensitivity in 10 strains of *Proteus vulgaris* it was found that these microbes were relatively sensitive. One strain was susceptible to 63 units of penicillin per ml, two strains were resistant to 32 units, another two strains were resistant to 16 units, while four strains showed resistance to 8 units of penicillin per ml.

2. In several experiments the growth of the bacteria was observed by direct agar microscopy. For investigation of the growth varying quantities of penicillin were added to the agar. On penicillin-free agar was seen growth of ordinary short or long bacteria. With very weak concentrations of penicillin (5 units per ml) there came growth of long and sometimes thicker threads, which did not become dissolved. On use of 13 units of penicillin per ml of agar there came growth of long threads with large bodies. After 6 hours' incubation incipient lysis set in, increasing on continued incubation. No increase in the number of the individual cells could be noted.

3. The occurrence of numerous large bodies is a prominent feature in the picture presented when *Proteus vulgaris* grows in media with weak concentrations of penicillin. These bodies seem to be more resistant to penicillin than the threadlike cell elements. They probably represent variation forms of the microbes.

4. Strains of *Proteus vulgaris* become considerably more resistant to penicillin when growing in media with increasing quantities of the antibioticum. During *in vivo* experiments with mice only a moderate increase of resistance was noted. The increased resistance was not attended by production of penicillinase. The resistant strains comparatively soon lost their increased power of resistance and again became sensitive when cultivated in penicillin-free media. In case of one particular strain it was found that the increase in resistance was not associated with loss of virulence.

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DIFFERENTIATION OF BETA STAPHYLOLYSIN IN TWO ANTIGENICALLY DIFFERENT COMPONENTS*)

By *Eigil Hess Thaysen.*

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Filtrates of cultures of pathogenic staphylococci are able to hemolyze erythrocytes from a number of animal species, and during the last decades it has been demonstrated that at any rate not all the hemotoxic properties of such filtrates are due to a single substance.

Thus, in 1935, Glenny & Stevens showed the existence of two antigenically different lysins: alpha lysin and beta lysin. Besides, alpha lysin was thermolabile and able to hemolyze, among others, rabbit and sheep blood — the latter though only at 37°. Beta lysin was thermostabile and able to hemolyze sheep blood in connection with the »hot-cold« phenomenon (see titration of toxin), while it was inactive for rabbit erythrocytes.

Previous attempts at further characterization of these lysins through a possibly present acute lethal and dermonecrotic effect are now in part given up, as it has been found that in all probability we here are faced by a number of mutually independent toxins. Alpha lysins, is of more frequent occurrence, either alone or together with beta lysins, and only in quite a few cases are the strains found to produce beta lysin almost exclusively (Forssman 1936; Bryce & Rountree 1936). In 1936 Morgan & Graydon thought they were able to demonstrate two lysins of the alpha type α_1 and α_2 — in nearly all their toxic staphylococcus cultures. Usually α_2 lysin was represented but scantily; yet, through its presence, it would considerably influence the titration of alpha antitoxin-containing sera as antigenically it differed from α_1 lysin. As yet we do not know with certainty whether these two toxin components, which originally

*) Supported by a grant from frk. P. A. Brandt's legat.

were isolated by precipitation of ordinary alpha toxin, occur in infections in vivo. On this account we cannot entirely exclude the possibility that they both have developed from the same original strain under the influence of the cultural conditions.

By means of a weak, dermonecrotic, strain (B_2) isolated by Forssman in 1938 *Llewellyn Smith & Price* (1938) obtained a lysin for rabbit erythrocytes, designated as gamma lysin. This lysin is extraordinarily thermolabile and it has a rather marked lytic effect on human blood which, according to *Roy* (1937) is very resistant to the ordinary alpha lysin. Gamma antilysin has been demonstrated in normal sera. It has not been practicable to demonstrate a possible identity between gamma lysin and *Morgan & Craydon's* alpha₂ lysin as, regrettably, these investigators have not placed any alpha₂ provisional standard serum at the disposal of other investigators.

At any rate two lysins of the alpha type are known, whereas hitherto the beta type has not been differentiated into various components. As it was realized even at an early date that there is no parallelism between the beta lysin contents of the staphylococcal toxins and, on the other side, their contents of leucocidin, acute lethal and dermonecrotic factors, this has undoubtedly contributed to slacken the interest in this type of lysin. In connection with the present work however, it is to be emphasized that *Minett* (1936) has isolated a number of staphylococcal strains pathogenic to dog that produced a strong beta lysin and almost no alpha lysin. On titration of this lysin with known sera Minett found so great divergences between the calculated values and those obtained that at first he mentioned the possibility of being faced by a new antilysin. In the same work, however, he gave up this hypothesis, among other reasons, because the sera employed were found to be very slightly beta antitoxic, on which account he assumed that the large amounts of serum required at the neutralization brought about the remarkable result through unspecific acceleration of the hemolysis.

In this work we have been able to demonstrate a beta lysin in staphylococcus of canine origin with specific antigenicity.

Strains.

During 1943-44, in routine investigation on staphylolysin, we received 18 staphylococcus strains which were found in pure culture in furunculosis in dogs. All these strains formed characteristic smooth white colonies on agar and were gram-positive. (C indicate canine strain). Only a few strains, including C₂, fermented mannitol and thus had to be designated by aureus. Filtrates of broth cultures of all 18 strains proved in varying degree to be beta lytic, whereas they were able to hemolyse rabbit blood but slightly or not at all. Unfortunately these experiments had to be discontinued for about nine months, and after this only 5 of the original strains — all stored in agarstabs at room temperature — were still alive. Further, of these five strains only two (C₁ and C₅) were still able to produce a fairly large amount of lysin, while one (C₃) produced the lysin but irregularly and scantily, and two

strains (Cs and C₁₁) lost their capacity for lysin production shortly after the experiment had been resumed. It was not practicable to reactivate Cs and C₁₁ through rabbit passages. Even relatively large quantities of concentrated bacterial suspension had no acute lethal effect on the animals. Blood cultures made 24 hours after the injection of the suspension turned out negative.

Technic for toxin production.

The culture medium employed for the lysin production was semifluid agar free from sodium chlorids and consisting of beef broth with 1 % Witte peptone, 0.2 % secondary phosphate and 0.2 % agar (pH = 7.5). As, according to Walbum (1922), any fairly large amounts of sodium chloride inhibit the lysin production, none was used in this medium. Like various other authors (e.g. Burnet (1930 and 1931), Parish & Clark (1932) and Bigger (1933)), we found that the addition of agar to the medium increased the yield of lysin considerably. On the other hand we were not able to decide whether the agar — as emphasized by Birch-Hirschfeld (1933) and McLean (1937) — plays any role as adsorbent of substances in the broth that inhibit the toxin production.

From the thorough studies reported by Walbum (1922), it is evident that the lysin production is greatest in neutral or slightly acid media (Parker, Hopkins & Gunther (1926)) emphasized the importance of lowered aerobiosis and Burnet therefore recommended to lower the partial oxygen tension by adding 10 % CO₂ to the atmosphere in which the bacteria are grown.

Therefore we have carried out the lysin production in closed containers, in an atmosphere with 10–40 % CO₂. After the incubation pH was found to be about 7.0, with rather wide variations regardless of the initial pH of the medium. Under entirely aerobic conditions pH soon adjusts itself to values between 8 and 9 and we have obtained only weak lysins in contrast to some investigators (Nelis (1934)). Furthermore, the lysin production appears to depend also on the proportion between the amount of medium and its surface. In the the present studies we have used Petri dishes with a diameter of 25 cm, and we obtained the best results with 50 ml. medium in each dish.

With the ordinary staphylococcal strains the optimum period for the lysin production is 5–7 days' incubation, whereas for the dog-pathogenic strains the curve for the lysin production is declining markedly already at this point of time, the yield being greatest and most uniform after about 3 days' incubation. The toxin was sterilized by filtration of the substrate first through filter paper in the Büchner funnel and then through Seitz filter. The lysins were stored at 4°. In the following experiments no lysin older than one month was employed.

The titrations of toxin and toxin-antitoxin.

These titrations are performed in the usual way with 1 % washed blood corpuscles as indicator in a total volume of 2 ml. Washing of blood corpuscles and dilution was performed with physiological saline. These suspensions were used within 1–2 hours after their preparation. Lysis of rabbit erythrocytes is read after 1 hour in water-bath at 37°, while lysis of sheep corpuscles is read after 1 hour in water-bath and standing for 1 hour at 4°. The endpoint of the titration is set at trace of hemolysis. As it is well known the blood corpuscles from different sheeps may vary much in their sensiliveness to the lytic action, on account of this the results of the different experiments cannot be compared directly. In order to save place the figures for the controls are omitted in all tables.

Table 1.
 Determinations of the Minimal Hemolytic Dose (M. H. D.)
 for the Toxins Employed.

Toxin	M. H. D. in ml. toxin			
	Erythrocytes			
	Sheep	Rabbit	Dog	Human
C ₁	0.002	> 0.5	> 0.5	0.004
C ₁ 60° for 30 min.	0.008			0.016
C ₂	0.03	> 0.5	> 0.5	
C ₂ (60° for 30 min.)	0.05			
C ₅	0.002	> 0.5	> 0.5	0.004
C ₅ (60° for 30 min.)	0.016			0.016
C ₈	0.25	> 0.5	> 0.5	
B ₈	0.001	0.001	0.002	0.004
B ₈ (60° for 30 min.)	0.004	> 0.5	> 0.5	0.016

The lysin production, expressed by the M. H. D. values, obtained with the remaining dog-pathogenic strains, is recorded in table 1. In the first experiments, strain B₈ was employed for controls, after heat treatment. This strain originates from a bovine mastitis, and, like nearly all bovine strains, it is a powerful alpha and beta lysin producer. As will be noticed, the filtrates of the dog-pathogenic strains are all beta lytic, in varying degree, whereas even ½ ml. undiluted toxin in no instance is able to give any alpha lysis. Like the lysis of the control, the lysin produced by these strains is thermostabile and also, in connection with a hot-cold phenomenon, able to hemolyze human blood but not canine blood. Finally, with a view to the following experiment, it is to be emphasized that the alpha lysin of the control is completely absent after heat treatment.

Serum neutralization.

The relatively powerful beta lytic filtrates from C₁ and C₅ were investigated further by means of serum neutralization. The culture

Table 2.
 Determination of LH/5 for Toxins C₁, C₅ and B₈ by Titration
 with Standard β-Antitoxin.

Toxin	ml. toxin ~ LH/10 Sheep blood	LH/10 M. H. D.
C ₁	0.008	2
C ₅	0.016	4
B ₈	0.06	8

Table 3.

Determination of LH/5 for Toxins C₁, C₅ and B_s by Titration with Standard β -Antitoxin.

Toxin	ml. toxin ~ LH/10 Sheep blood	LH/10 M. H. D.
C ₁	0.025	6.3
C ₅	0.020	5.0
B _s	0.16	20.0

filtrate of B_s served as control, and the serum here employed was the provisional *standard beta antitoxin* (Ipsen & Rostock) (1946) which per ml. contained 20 provisional beta units, < 1 international alpha unit and 7 provisional gamma units. First LH/10 was determined for this antitoxin, with the ratio 1 : 2 between decreasing toxin dilutions. This orientating titration showed a difference in the neutralization effect of the serum on the beta lysin of the two canine strains and of the control. In a subsequent, more precise, titration with only 25 % interval between the toxin dilutions the LH/5 value were performed for the same lysins. (Table 3).

From table 3 it will be noticed that $\frac{1}{5}$ unit of standard beta antitoxin is 3—4 times less active against the lysin of C₁ and C₅ than against the lysin of the control. From table 4 it is also evident that the serum is less specific for the lysin of the canine strains — for which the neutralization curve is relatively flat — than for the lysin of the control, for which the curve is very steep. A fixed dose of toxin —namely the dose which, according to table 4 gives total or subtotal

Table 4.

Course of Neutralization for Toxins C₁, C₅ and B_s, Titrated with $\frac{1}{5}$ Unit Standard β -Antitoxin.

Toxin titrated with $\frac{1}{5}$ unit standard β antitoxin	C ₁	C ₅	C ₈
0.16 ml.	+++	+++	+++
0.125 "	+++	+++	0
0.100 "	+++	+++	0
0.080 "	+++	+++	0
0.063 "	+++	+++	0
0.050 "	+++	+++	0
0.040 "	++	+++	0
0.032 "	+	++	0
0.025 "	(+)	+	0
0.020 "	0	(+)	0
0.016 "	0	0	0

+++ = Total hemolysis
 ++ = subtotal "
 + = partial "
 (+) = slight "
 0 = no "

hemolysis with $\frac{3}{5}$ unit standard beta antitoxin — was added to varying dosis of this serum, and the amount of beta units required to neutralize the 3 lysins was determined. Since 1 unit neutralizes 5 times more M. H. D.'s of the control beta lysin than of the canine lysins, this experiment indicated a difference in antigenicity.

Table 3.

Titration of Toxin from the Dog-pathogenic Strains and from the Control with Provisional Standard β -Antitoxin.

Toxin	No. of β standard units required for the neutralization	M. H. D. \sim 1 unit β standard
C ₁ (0.04 ml.)	0.25	40
C ₅ (0.032 ml.)	0.20	40
B ₈ (0.16 ml.)	0.10	200

Titration of homologous immune sera.

Rabbits 7854 and 7855 (whose normal serum was not antilytic) were immunized respectively with C₁ and C₅ toxin. In 3 weeks the animals were each given 5 intravenous injections of unheated toxic filtrates in doses increasing from 0.2 to 2 ml. The animals were quite unaffected by this treatment. 8 days after the last injection, blood was taken for the preparation of immune sera 7854-C₁ and 7855-C₅. The control employed in this and the following experiments was the beta test lysin γ 17, with which the standard antitoxin is produced, and which is generally used in this department for determination of the beta antitoxin contents of a given serum. Further this lysin proved as well antigenically as after the method of Ipsen to be described later in this paper to be identic with the beta lysin of strain B₈. The toxins were titrated, using serial semidilutions against 1/10 ml. serum 7854-C₁ and 1/500 ml. serum 7855-C₅, respectively (Table 6). Both lysins were cross-neutralized to the same extent by the immune sera produced with the homologous and the heterologous strains. The two sera had only a very slight effect upon the beta test toxin. This experiment showed conclusively that there are two lysins of the beta type — Glenny's classical beta toxin — in the following designated as beta₁ — which constitutes the main part of the lysin in toxins B₈ and γ 17, while the other designated as beta₂ is represented in these toxins but scantily. In contrast hereto, lysin of the canine strains contains predominally beta₂ and merely a trace of beta₁. With the view to further investigation, on the basis of the results recorded in Table 6, 1/100 ml. serum 7855-C₅ was defined as one provisional beta₂ unit. This unit neutralizes about 160 M. H. D. beta₂ lysin.

Table 6.

Titration with Immune Serum for the two Dog-pathogenic Strains.

Toxin	No. of M. H. D. neutralized by 1/10 ml. serum 7855—C ₁	No. of M.H.D. neu- tralized by 1/500 ml. serum 7855—C ₅
C ₁	30	32
C ₅	30—50	32
Y ₁₇	ca. 1	2—3

Titration of random horse sera.

It is evident that the presence of even small, but varying, amounts of beta₂ antilysin in the different sera will be able to influence the determination of their contents of beta antitoxin when the composition of the test toxin is unknown. In order to get a preliminary idea of the significance of this fact, the neutralizing effect of the standard beta antitoxin and two equine immune sera (1384 and 2035) on the beta₁ toxin (y 17) was compared to that of the two lysins of the canine strains. The results are recorded in Table 7, where a fixed dose of toxin *i. e.*, the dose which, according to the preceding experiments, just is neutralized completely by $\frac{1}{5}$ unit of standard beta₁ antitoxin — is titrated against decreasing amounts of serum (25 % variation).

Table 7.

Comparison between the Neutralizing Effect of Standard β -Antitoxin and of 2 Immune Sera, on β -Lysin in Toxin Y₁₇ and in the Toxin of 2 Canine Strains, expressed in Provisional Standard β -Units.

Toxin dose	C ₁ 0.02 ml.	C ₅ 0.016 ml.	Y ₁₇ 0.14 ml.
Standard β -antitoxin ml.	0.008	0.0063	0.0125
" " β -units	0.16	0.125	0.25
Serum 1384 ml.	0.00125	0.001	0.0022
" " No. of standard β -units per ml.	128	125	114
Serum 2035 ml.	0.01	0.008	0.04
" " No. of standard β -units per ml.	16	15.6	6.25

From Table 7 it will be noticed how many standard beta₁ units or how large quantities, of the two sera are required for the neutralization of the individual lysins in the given doses. In order to obtain a basis for comparison, from these values the potency of the two sera is calculated for each toxin, expressed by the number of standard beta₁ units per ml. serum. Serum 1384 seems to be considerably more potent than the standard antitoxin, and the increase in potency is about pro-

portional for all 3 toxins (128, 125 and 114 units). Thus the antilysin distribution of serum 1384 must be about the same as in the standard antitoxin. On the other hand, the weaker serum 2035 shows no such consistency the serum being 2.5 times more potent against β_2 toxin than against β_1 toxin.

The following experiment aimed at a preliminary estimation of the amounts of the two beta antilysin components in some sera. No pure β_1 and β_2 antilymins were available but we utilize the fact that in the titration of a mixed serum against two toxins that act on the same indicator system, it usually will be possible to determine the strength of the component which is more weakly represented if only the relevant toxin is present in excess. In this titration we assumed that only two beta lysins exist. As is evident from table 8, the wood toxin, the β_1 test toxin and the toxins of the canine strains represent just such bacterial toxins where one lysin component is known beforehand to be represented preponderantly. Besides the above-mentioned sera, also a pooled normal dog serum was examined. The results are recorded in table 8, given in units of the respective antilysin per ml. serum.

Table 8.

Preliminary Estimate of the Antilysin Distribution in a Number of Sera.

	Red blood cells	1. ml. stand. anti- α toxin	1. ml. provisional stand. β_1 antitoxin	1. ml. provisional stand β_2 antitoxin	1. ml. serum 1384	1. ml. serum 2035	1. ml. pooled normal dog serum
Wood toxin ($\alpha > \beta$)	rabbit	20 α U.*)	<1 α U.	ca. 1/10 α U.	80 α U.	60 β U.	1,10 α U.
test toxin ($\beta_1 > \beta_2$)	sheep		20 β U.*)	2 β U.	114 β U.	5 β U.	2 β U.
β_1 toxin ($\beta_2 > \beta_1$)	sheep	>1/10 β_2 U.	7.5 β_2 U.	100 β_2 U*)	50 β_2 U.	6 β_2 U.	10 β_2 U.
β_2 toxin ($\beta_2 > \beta_1$)	sheep	>1/10 β_2 U.	7.9 β_2 U.	100 β_2 U*)	50 β_2 U.	6 β_2 U.	10 β_2 U.

*) by definition.

It will be noticed that both lysin components are present in all the beta antitoxin-containing sera and that their mutual ratio varies considerably. The provisional β_2 antitoxin contains a relatively large amount of both beta antilymins, while the contents of alpha lysin is strikingly low. A similar antilysin distribution was subsequently found also in all the other sera of normal dogs that we have examined. So there can be no doubt that the β_2 lysin is present in infections in dogs.

For control, finally, I have employed the method given by Ipsen (1940) for demonstration of lysin relationship. When in a coordinate system we plot the logarithms of the corresponding times (log. T), we obtain a reaction curve (dose-time curve) that has proved to be quite specific of each lysin. (As to particular details, the reader is referred to the original work of Ipsen.)

Fig. 1 shows the curves for the beta test lysin and beta₂ lysin. The curve for beta₂ lysin is obtained by employment of C₅ filtrate, which contains this toxin almost exclusively.

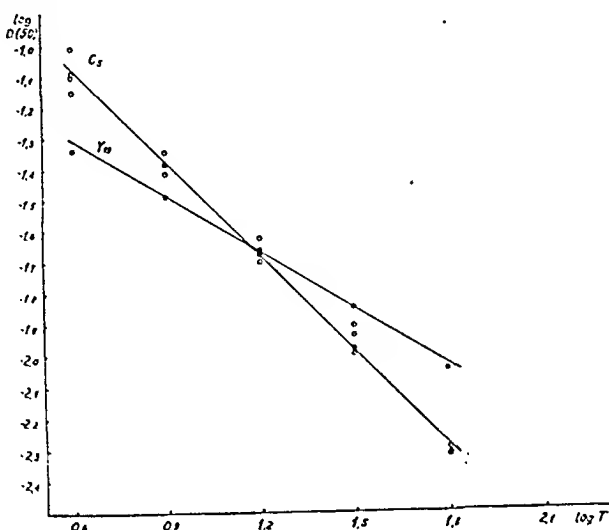


Fig. 1.

It will be noticed that both curves within the given periods (4—64 min.) are approximately linear, and that the inclination of the curve is also dependent upon the lysin employed. The inclination coefficient of the curves of the time exponent alpha can be determined as $\frac{\Delta \log. T}{\Delta \log. D 50}$. The calculated values are given in table 9 — the value for the alpha lysin is taken from Ipsen's work.

Table 9.

Time Exponent α for Staphylo-lysin.

α is increase in log. time for decrease in log. of dose causing 50 % hemolysis.

α lysin	B ₁ test lysin	B ₂ lysin
1.25	1.68	1.08
	1.53	1.00

Here we find that the time exponents for the alpha lysin, beta test lysin and beta₂ lysin mutually differ considerably.

Discussion.

It has been established that there exist at least two antigenically different lysins of the type beta (beta₁ and beta₂) and that they both occur side by side and in mutually varying quantitative proportion in

the beta toxic filtrates here examined, directly or indirectly. The filtrates of the strains C_1 and C_2 are almost entirely beta₂-lytic and contain practically no alpha lysin.

After the conclusion of this work, we have received from Stockholm and England several staphylococcus strains, recently isolated from furunculosis in dogs. These strains had all to be characterized as *Staphylococcus albus* and in an orientating examination most of them were found to form a powerful beta₂ lysin, and only in a few cases negligible quantities of alpha lysin were produced. From Stockholm we were also informed that previously the cultures from furuncles in dogs practically always yielded staphylococci forming white colonies on agar. It looks as if the ordinary alpha toxin-containing aureus strain play no pathogenetic role in dogs. But this question needs further investigation, especially by experimental infection of dogs.

Finally, we have examined staphylococci isolated from a number of other animals (horse, cow, sheep, rabbit, hare, beaver) with a view to the lysin production. In no instance could any new lysins be demonstrated.

Summary.

In filtrates of staphylococcus cultures originating from furunculosis in dogs, a lysin of characteristic beta type (beta₂) has been demonstrated. By its antigenic properties and its specific dose-time curve ad modum Ipsen this lysin differs from the beta₁ lysin. Both lysins occur side by side in the ordinary beta-toxic filtrates examined so far. Normal dog serum contains relatively large amounts of beta₂ anti-lysin. A provisional beta₂ unit is defined.

I wish to thank Dr. R. St. John-Brooks, National Collection of Type Cultures, Lister Institute, Elstree; Laborator Per Vividén, Stockholm and the Royal Veterinary and Agricultural College, Copenhagen for their valuable assistance in providing me with dog pathogenic strains.

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PARABIOSIS AND RESISTANCE TO TRANSPLANTATION. II

THE INFLUENCE OF PARABIOSIS ON THE GROWTH OF A MOUSE CARCINOMA AND A MOUSE SARCOMA IN IRRADIATED RATS*)

By *Jorgen Bichel* and *Ib Holm-Jensen*.

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In a previous communication (Bichel and Holm-Jensen, 1947) the authors studied the transference of resistance to tumour transplantation between parabiotic animals.

The experimental animals, Wistar rats, were coupled together two and two to form parabiotic pairs according to the method of Bunster & Meyer. One or both parabions were irradiated all over with a dose of X-rays sufficient to make 100 per cent of single control animals temporarily susceptible to the transplantation of a mouse leukemia forming local tumorous infiltrations. The leukemia was grafted into one or both of the parabiotic animals.

These experiments revealed that the susceptibility of the irradiated rats to transplantation of the mouse leukemia would always be completely abolished if the irradiated animals were united in parabiosis with non-irradiated partners, whereas the resistance of the non-irradiated parabions was never influenced by the parabiosis.

In consequence of investigations carried out by Harris (1943) we may take it for granted that resistance to heterologous transplantations generally is not due to a primary resistance, but is dependant upon an induced immunity only.

When the resistance to heterologous grafts once has been induced, it apparently cannot be broken down by irradiation. This was originally shown by Clemmesen (1938) for grafts of mouse sarcoma 180 in

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rats, and was confirmed by the present writers who found the same for the resistance induced in Wistar rats by the mouse leukosis in question.

In view of the above facts our experimental results probably indicate that the transference of resistance from the non-irradiated to the irradiated animal in the parabiosis is due to antibodies (against mouse tissue) which are called forth in the non-irradiated parabion by antigenic substances, which as well as the antibodies produced must be able to pass from one parabion to the other.

As these experiments were carried out with a mouse leukosis, which often, even in the irradiated rats becomes generalized, it could not be ascertained whether the resistance which was induced in the non-irradiated parabion was evoked by transferred living cells or by substances derived from the graft.

To elucidate this point further we have carried out a number of experiments with two mouse tumours which show very little tendency to metastasize. One tumour was a transplantable mouse carcinoma, the other a sarcoma. Previous experiments were made to make sure that none of these tumours would grow in normal Wistar rats, whereas temporary growth would take place in rats which had been irradiated all over with 630 r.

The experiments were carried out exactly as the experiments on the mouse leukosis to which reference regarding technique and experimental conditions may be made.

1. *Experiments with a mouse carcinoma.*

The mouse carcinoma was a *carcinoma solidum* originating from a mammary carcinoma (Krebs II mouse carcinoma) which has been kept in this laboratory for many years. It is a rather slow-growing tumour with a tendency to necrotize in the older tumours. Metastases in the mice are rare.

The following experiments were carried out:

1. Two Wistar rats were united in parabiosis. A few days later one animal was irradiated all over with 630 r (the other animal being screened off with lead) and 24 hours later, the irradiated animal was grafted with the mouse carcinoma. Simultaneously the tumour was grafted into two single control animals, one irradiated the other non-irradiated. (Exp. nos. 28, 35, 39).

Results: Takes of the tumour in all the irradiated control animals. No takes in any of the other animals.

2. Carried out as the foregoing experiment; the only difference being that the tumour was now grafted into both parabions. (Exp. nos. 37, 38).

Results: As in 1.

3. Carried out as experiment 1, with the difference that the tumour was now grafted into the non-irradiated parabion. (Exp. nos. 62, 81).

Results: As in 1.

4. After parabiosis had been established for some days, both rats were irradiated and tumour was grafted into one of the parabions and simultaneously into a single irradiated and a single non-irradiated control rat also. (Exp. nos. 102, 103).

Results: Takes were obtained in the grafted parabions and in the irradiated control animals.

II. *Experiments with a transplantable spindle cell sarcoma (Oppau).*

This tumour is a fast-growing cellular spindle cell sarcoma, very rarely metastasising in the mice.

The experiments were carried out in the same way as the above experiments with the mouse carcinoma.

(1 corresponds to I 1 whereas 2, 3 and 4 correspond to I 2, I 3 and I 4).

1. Exp. nos. 13, 14, 51, 54 and 55. Results as in I type 1.

2. » » 15, 16 » » » I » 2.

3. » » 82, 83 » » » I » 3.

4. » » 90, 91 » » » I » 4.

5. In these experiments both animals in the parabiosis were irradiated and both of them inoculated with the tumour. Control animals as usual (Exp. nos. 17, 18 and 19).

Results: takes in both parabioses in exp. 18 and 19 and in the irradiated single rats. In exp. 17 take was only obtained in one of the parabioses.

These experiments clearly demonstrate that even tissue-grafts which ordinarily do not metastasize, when grafted into one parabion may cause a production of antibodies in the other parabion.

This finding strongly supports the assumption that the immunity in question is not dependant upon the transference of living cells. So we have to assume that the immunity met with should be ascribed to metabolites or disintegration-products from the inoculated cells.

Summary.

Two mouse tumours, a carcinoma and a sarcoma, will grow temporarily in irradiated rats. If the irradiated rats are coupled in parabiosis with non-irradiated animals, their susceptibility to transplantation of the mouse tumour will be abolished. The results are similar to those previously obtained with a mouse leukaemia, but as the mouse carcinoma and sarcoma have no tendency to metastasize, the present experiments probably indicate that the induced resistance to transplantation of tumours may be developed as a result of an action of cell metabolites or disintegration products.

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ON THE PATHOGENESIS OF APPENDIXACTINOMYCOSIS

By Lennart Zettergren.

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Apparently, the question of the systematics of the actinomyces still remains to be solved. As yet, no generally acceptable classification has been presented. *Bergey* made a rough differentiation into four families, viz., the leptothrix, the crysipelothrix, the proactinomyces and the actinomyces. The types relating to the *leptothrix* form long, gram-positive fibres and seem to occur, more or less saprophytically, in, inter alia, the oral cavity, particularly at angina, stomatitis and gingivitis. They have also been observed in old, callous gastric ulcers. The form referring to the *crysipelothrix* gives rise to dysentery in pigs, while another belonging to the same family not infrequently causes the so-called crysipeloid manifested especially by persons often dealing with raw meat. The *proactinomyces* group includes numerous aerobic actinomyces occurring in nature. Finally, the *actinomyces* comprise the fungous form which, in the majority of cases (approx. 70 per cent), produces the typical actinomycosis in Man. Fungi relating to the last-mentioned family occur, to a large extent, saprophytically in the oral cavity of the human being.

Actinomycosis represents one of the more rare diseases. In Sweden, about 80 new cases are calculated to appear annually, the majority being of the cervico-facial type as regards the localization. In a comprehensive statistical survey from Finland (*Brofeldt*), the distribution among the most common localizations is as follows:

Cervico-facial localization	58.4	per cent.
Abdominal localization	32.4	» »
Thoracopulmonal localization	17.2	» »
Cutaneous actinomycosis	2.0	» »

The abdominal infection is, in most instances, localized to the ileocecal region where it gives rise to a palpably solid granulation tissue, at the clinical examination often giving the impression of a tumour fixed to the abdominal wall. The chronic course of the infection is characteristic, with a marked tendency to the formation and involvement of adjacent tissues and organs. However, judging from the literature in this field, a primary localization in the appendix seems to be exceedingly uncommon. Out of the few reliable observations made on this point, those published by *Bevan* (1904) and *Kaplan* (1923) may be mentioned here. They found no foreign bodies in the appendix.

The present author has been in a position to study more closely a case of primary appendixactinomycosis which may be of some interest in the discussion regarding the intestinal actinomycosis. Furthermore, since it does not appear to have any parallel in the literature, a brief account of this case seems motivated.

H. J. A lumberer. Aged 32 years. Treated at The Surgical Department of the Falu Hospital during the period of September 9th—October 21st, 1916, under the diagnosis of *Appendicitis chron.* (case record no. 2678/46). At Midsummer of the same year, the patient fell ill with a pain in the pit of the stomach. It was periodically very intense, often lasting for an hour or two and extending diffusely in the abdomen. After about a month, the pain concentrated mainly to the right fossa iliaca. He was affected with nausea in connection with the pains but did not vomit. No chills. A fair amount of noise and rumbling in the abdomen. No troubles at evacuation. At an examination on August 8th, 1916, a well-delimited tumour, the size of half a fist, was found in the right fossa iliaca, being clearly mobile. Sedimentation rate: 43—50 mm/1 hour. At admission to the Falu Hospital ten days later, the patient had an unaffected general condition. Temperature: 37.4—38.0°. Lungs and heart: without remark. Bl. pressure: 145/100 mm Hg. Abdomen: Soft but tender at deep palpation in the right fossa iliaca. No definite resistance was palpated. Per rectum: Distinct tenderness in the right fossa Douglasi. Blood: Hg 90 %. White bl. corp. 11000, 1600 of which were mononuclear. Sed. rate: 43 mm/1 hour. *Röntgen exam.*: Valv. Bauhini lets the contrast matter through, the last ileum loop disclosing an excellent mucous membrane. The cecal pole not completely filled owing to a resistance, situated medially and below the pole. This resistance felt tender, causing a suspicion of an old appendicitis abscess (*Saul*).

Operation (Tolagen) on Sept. 28th, on a suspicion of a tumour or inflammatory process in the cecal pole: *Appendectomy + resectio caeci*. Extensive Mac Burney incision in the right fossa iliaca. It was found that the process constituted a chronic phlegmonous appendicitis where the solid, fibrotic appendix, the thickness of a finger, is baked into tough fibrotic areas between the small intestine and the caecum. When exposed, the appendix was seen to have been perforated approximately in the middle where an abscess cavity was found, the size of a Spanish nut, filled with messy granulations. The appendix was removed by means of diathermy, as well as the surrounding fibrotic parts of the caecum. Primary suture. — A moderate rise in the temperature set in during the days following the operation. At a control examination three weeks after his discharge, he stated that he had been free from troubles the whole time. Sed. rate: 6 mm/1 hour. *P. A. D. (Gellerstedt)*: Appen-

ditis chron. (tb. appendicitis?) + Appendicolithiasis actinomycotica. A complicated case with interesting changes. The distal half of the appendix disclosed a chronic-inflammatory, fibrous thickening of the wall, especially in the external layers. In the basal part of the appendix, the wall was thinner but with marked inflammatory changes, partly with purulent manifestations of dissolution, as well as, in several places, minor tubercle-like foci in the



Fig. 1.
Appendix concrement
in natural size.

mucons membrane and the submucosa. In the middle, the appendix lumen was slightly dilated, containing, apart from pus, also a concrement, hardly the size of a pea and resembling a spiked club, which is an uncommon formation in this particular part (Fig. 1). The mucous membrane in the last-mentioned part disclosed purulent dissolution. In the pus surrounding the



Fig. 2.

Section of the concrement revealing a central nucleus and a peripheral stratified part. Gram-Weigert stain, x 20.

stone a mass of small fungus colonies was observed, consisting of a gram-positive, ramified mycelium. Thus, it was definitely established as a case of actinomyces. Fungal mycelium of a similar appearance was also to be seen in the purulent and dissolved mucous membrane. — *Examination of the concrement (Zettergren):* The stone was embedded in celloidin and decalcified in a 5 per cent solution of trichloroacetic acid. After carefully dissolving the celloidin and renewed embedding in the same material, the stone was subjected to serial sectioning and stained according to Gram-Weigert's method. Some of the sections were submitted to connective tissue staining. — The centre of the stone was found to consist of a nucleus which was fairly well delimited from the peripheral part which had a concentrically stratified structure (Fig. 2). In the nucleus of the stone, here and there small vegetal fragments were seen. In one place such a fragment lay in close contact with vegetations of a typical actinomyces mycelium (Fig. 5). This was also to be noticed in several places in the centre of the stone, often radiating towards the surface of the external layer (Fig. 4). However, the occurrence of mycelium was here relatively sparse, only to increase strongly in the likewise stratified, wart-like offshoots (Fig. 3). The delicate mycelium was gram-positive and ramified throughout. No club-like formations were seen. In the nucleus of the stone, preparations of stained connective tissue disclosed fragments with distinct fibroblast nuclei. In the periphery of the stone, isolated gram-positive rods and cocci occurred, of varying size and shape.

Summary: A man, aged 32, fell ill with signs of appendicitis. At operation, the appendix was found to be the site of a chronic, phlegmonous inflammation with perforation. Dissection of the surgical preparation revealed a concrement in the appendix lumen, the size of a pea and



Fig. 3.

Detail of one of the wart-like offshoots of the stone showing the stratified vegetations of the actinomyces. Gram-Weigert stain, x 50.



Fig. 4.

Fungous mycelium in concentric growth zones in the nucleus of the stone.
Gram-Weigert stain, x 400.

resembling a spiked club. The microscopical examination disclosed the occurrence of a chronic appendicitis with actinomyces mycelium, partly in the pus, and partly in the mucous membrane which had undergone purulent dissolution. The concrement consisted of a nucleus and a peripherally stratified part, both containing actinomyces mycelium. In addition, in the central part of the stone, vegetal fragments were observed as well as a connective tissue with quite distinct fibroblast nuclei.



Fig. 5.

Fungous mycelium lying in direct surface contact with a vegetal
fragment. Gram-Weigert stain, x 870.

Discussion.

As regards the pathogenesis of the actinomycosis disease, opinions seem to differ. Earlier, the human being was supposed to become infected by chewing grass, chaff or similar plants. Investigations by *Schwarz* and *Söderlund*, among others showed that, for instance, grains of corn or parts thereof have a noteworthy effect on the production of actinomycetes of the salivary glands. However, the fungi found on these plants are aerobic, whereas actinomycetes of this type have been observed in but a few instances in cultures from actinomycotic granulation tissue or from salivary stones (*Naeslund*). Accordingly, the actinomycetes occurring in nature are not, apparently, guilty to any marked degree of communicating the actinomycosis disease to Man. Nor does direct transmission from infected persons, or from animals to human beings, seem to be of any significance with regard to the origin of this disease. Still, it has been possible to cultivate anaerobic actinomycetes from deposits of dental and tonsillar calculi. Moreover, pulmonary actinomycosis has been known to develop occasionally in connection with the aspiration of tooth stumps. These observations serve to indicate that the generally saprophytic actinomycetes occurring in the oral cavity should, in the first place, be considered as facultatively pathogenetic with regard to the carrier. Since these fungi are, however, extremely common in the oral cavity of healthy persons, the forementioned hypothesis seems to presuppose an increase in their virulence under suitable conditions. This may happen owing to a general or local deterioration in the power of resistance of the organism. Nevertheless, vegetal fragments undoubtedly play an important part in communicating an actinomycotic infection, not merely by serving as vehicles for the saprophytic actinomycetes in the oral cavity but also owing to the fact that they may cause the injury to the mucous membrane in preformed canals (such as the salivary ducts, the bronchi, the intestinal canal, including the appendix) which is a prerequisite for the penetration of the fungi into the tissue and the subsequent development of the inflammatory process.

In the present case, it seems justified to regard the vegetal fragments occurring in the nucleus of the stone as the vehicle and conservative substratum of the fungal mycelium, particularly since mycelian growth was ascertainable in direct surface contact with a few vegetal particles. Furthermore, the fact that fungal colonies were most abundant in the pus surrounding the stone renders probable the assumption that growth took place centrifugally, with the stone nucleus as the starting-point, and also that the symptoms had set in at a time when the fungus had reached the appendix lumen and its toxins had caused inflammatory irritation. However, it remains to settle the extent to which the actinomycosis can be regarded as responsible for the actual appendicitis. This is a complicated matter in view of, *inter alia*, the generally abundant intestinal flora in the appendix and be-

cause pure cultures of the actinomyces are never seen in the pus from actinomyeotic foci. This latter circumstance was observed by *Klinger* (1912), among others, and later verified by *Cloebrook*, *P. Holm* and *Naeslund*. Thus, the actinomyces are invariably accompanied, to a varying extent, by generally anaerobic bacteria, such as streptococci, fusiform bacilli, and the so-called bact. actinomycetum concomitans. The part played by these bacteria in the course of the actinomyeotic infection has not been elucidated. Direct support for the assumption of the actinomyces as the etiological factor in the present appendicitis case is obtained from the fact that fungus colonies have been found not only in the pus surrounding the stone, but also, to a predominating extent, in the mucous membrane subjected to purulent dissolution.

The striking similarity between the concrement in the present case and in some salivary stones described by *Söderlund* should be noted. It is manifested partly in the occurrence of a homogeneous stone nucleus with peripheral stratification, and partly in the presence of vegetal particles in the interior of the stone. The conformity also refers to the shape of the stone and to the localization of the bacterial vegetations in the concrement. *Söderlund* gave an account of a salivary stone with wart-like offshoots which, as in the present author's case, probably derived from the calcium-precipitating capacity of the isolated actinomyces vegetations. All these similarities between the actinomyeotic salivary stones described by *Söderlund*, on the one hand, and the appendix concrement in the present case, on the other, offer further support of the, *a priori*, assumed genetic connection between the concrement and the inflammatory process in the appendix.

When attempting to explain the presence of the concrement in the appendix, this may be assumed either to refer to a salivary stone which has been swallowed and has penetrated into the appendix where it has stuck, or to an autochthonous calculary formation. However, staining of the connective tissue of the concrement showed that it contained, in the nucleus, a number of connective tissue fragments with distinct fibroblast nuclei deriving from the food. This would suggest that the appendix concrement should be an autochthonous product.

The question now arises whether the physico-chemical processes causing the actinomyeotic stones in the salivary glands can also take place in other organs. According to the opinion arrived at by *Naeslund* partly by experimental means, a salivary stone containing actinomyces appears in the following manner: The protein in the saliva is permeated in the neighbourhood of the growing fungus, involving on the part of the calcium salts in the saliva a reduction in the colloid protection which causes the precipitation of calcium salts. Simultaneously, a decrease in the pH takes place next to the fungus with a change in the stability of the colloid and its subsequent precipitation. The stratification typical of the salivary stone containing actinomyces oc-

rurs through an alternating accumulation of crystalloids and colloids. It may be asked to what extent an inflammatory exudate in, for instance, the appendix can offer conditions contingent to the formation of a concrement similar to that of the saliva. It goes without saying that the necessary amount of protein occurs in the exudate formed by the fungal toxins. In addition, it seems likely that salts also occur in sufficient quantities. Findings of appendix concretions, similar to that of the present author's case, (*Falkenstein, Rochaz*) and, in at least one instance, consisting of calcium and phosphoric acid (*Falkenstein*), justify the assumption that, under certain conditions, approximately the same prerequisites for the development of concretions are manifested in the appendix as in the salivary ducts. Unfortunately, no chemical analysis was made of the concrement in the present case.

Apparently, as regards the nature of the tubercle-like foci and their possible relation to the actinomycotic infection, no definite conclusions can be drawn. However, no acid-fast rods were found in the sections subjected to examination.

Summary.

The present author describes a case of appendix-actinomycosis in a man of 32 years of age. Operation revealed a solid, fibrotic appendix baked into tough fibrotic areas between the small intestine and the cecum. In the middle part of the appendix a perforation was seen and close to it an abscess cavity, the size of a Spanish nut. At dissection of the organ, a stone was found resembling a spiked club and hardly the size of a pea. The histological examination of the appendix disclosed chronic inflammation of this part. At the site of the stone, the lumen was filled with pus, as well as containing a number of actinomyces colonies. Fungal mycelium was observed also in the mucous membrane which had undergone purulent dissolution. This, according to the present author, speaks in favour of the etiological significance of actinomyces with regard to the occurrence of appendicitis. The concrement was found to consist of a fairly homogeneous nucleus and a concentrically stratified peripheral part. In the stone nucleus, here and there small vegetal fragments were seen as well as stratified vegetations of typical actinomyces mycelium, in some places in direct contact with each other. In the peripheral, concentrically stratified layers, the fungus in the isolated inner parts often grew in a radial manner towards the external surface layer. In the wart-like offshoots, which were also stratified, the mycelium was more abundant than elsewhere. In the present author's opinion, a genetic connection exists between the concrement containing actinomyces and the appendicitis. From the occurrence of a connective tissue, containing fibroblasts, in the stone nucleus, probably deriving from the food, the conclusion

can be drawn that the concrement has in this case formed in the appendix. Further, a striking resemblance is pointed out between certain actinomycotic salivary stones and the present appendix concrement. The possibility of similar physico-chemical processes, that give rise to actinomycotic stones in the salivary glands, is discussed, also in the present case of a concrement formation in the appendix.

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ENCAPSULATED SOLUBLE-HAEMOLYSIN-PRODUCING "TRUE R PHASE" (DAWSON) PNEUMOCOCCI OCCURRING IN THE HUMAN THROAT¹)

By Thorolf Packalén.

(Received for publication February 4th 1948.)

Brown's (1) classification of the streptococci in alpha, alpha prime, beta and gamma varieties according to their effect on the red cells in blood agar plates has attained general currency in bacteriological literature. Soluble haemolysins are produced only by streptococci belonging to the β -type, most regularly by those of Lancefield's serological groups A, C and G. The generally accepted inability of α -streptococci to form soluble lysis is contrasted with the almost regular formation of such a lysis by pneumococci (3, 14 a, 18, 25). Both of them cause a similar greenish discoloration of the blood agar.

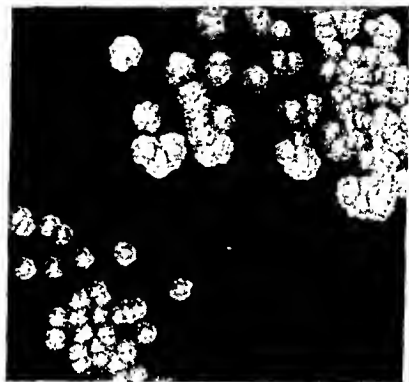
In practice the examination for soluble haemolysin may be made by direct filtration of the haemolytic activity of a 16 to 24-hour glucose-broth culture. Previous removal of the bacteria by filtration is superfluous.

When in an assay of the haemolytic activity of β -streptococci isolated from the throat of tuberculosis patients a few α - and α' -haemolytic strains were included as controls, it was quite unexpectedly observed that some of the latter completely haemolyzed the sheep blood, sometimes even in dilutions of 1 : 1,000 or higher. In the first tubes of the dilution series the haemolyzed cell suspension was mostly brownish discoloured, in addition sometimes developing a brown flocculent precipitate. This phenomenon is seldom seen in the β -streptococcal haemolysis. The haemolytic activity was sometimes unexpectedly lost at subcultivation, but as a rule it could be restored by further passages.

1) An abbreviated version of this paper was read before the Section of Hygiene and Bacteriology of the Swedish Medical Association, November 23, 1946.

Biological characteristics.

All soluble-lysin-forming, green-producing strains so far studied have shown, after aerobic as well as anaerobic incubation, their characteristic α - or α' -type of lysis in blood agar plates prepared of human, horse, sheep, or rabbit blood. On solid media the colonies were about 1 mm. in diameter, somewhat irregular in shape, and had a more or less rough surface. (Figs. 1 and 2).¹ They yielded extremely granular suspensions. In fluid media they grew as a loose flocculent sediment resembling a tuft of cotton at the bottom of the tube, leaving the supernatant layer clear.



Figs. 1 and 2.

Colonies of strains 961 and 1082 respectively after 3 days' incubation at 37° C.

The streptococci were often somewhat elongated (Fig. 3), sometimes however even compressed laterally, so that the longer axis of the cell lay at right angles to the axis of the chain. They had distinct though thin capsules, and were definitely Gram-positive when taken from young cultures. They grew in tangled masses of enormous chains and skeins (Fig. 3).¹



Fig. 3.

Gram-stained preparation of a 20-hour glucose-broth culture of strain 961. (X1000).

¹ For these photographs the author is greatly indebted to Dr. J. Fåhræus.

By passing the strains serially through rabbit-blood infusion broth twice daily for a few weeks, a diffuse growth with only insignificant sediment formation was eventually obtained. In spite of this the streptococci still formed rather long chains often consisting of up to 10—20 individual cells. The roughness of the colonies often became less pronounced, however, without changing into the S type. (In the following these strains will be designated as "adapted" strains).

Biochemically our green-producing strains behaved principally like streptococci of the salivarius group. Their resistance to bile was considerable, but under suitable conditions a definite clearing of the coccal suspensions occurred when mixed with a 10 % deoxycholate solution. A more detailed analysis carried out by Dr. Erna Moreh¹⁾ at the State Serum Institute in Copenhagen, gave the following results: Five and ten per cent solutions of sodium taurocholate killed the cocci within 10 minutes, a 2½ % solution only after 3 hours.

Optochin 1 : 50,000 in blood agar caused partial inhibition of their growth. The "adapted" strains were neither soluble in a 10 % sodium taurocholate solution even after 3 hours' incubation at 37° C, nor inhibited by optochin 1 : 50,000. They showed sometimes a tendency towards losing their haemolytic activity.

Serological characteristics.

Lancefield's group sera did not react with any of three strains so examined, viz., 961, 1082 and 1088.²⁾ Antisera prepared against these three strains agglutinated homologous as well as heterologous strains, though the former as a rule to a somewhat higher titre (Table 1). No capsular swelling was observed. Similarly crosswise performed absorption tests revealed a close serological relationship be-

Table 1.

Agglutination of the strains 961, 1082 and 1088 in homologous and each others' immune sera (prepared by immunizing rabbits three times weekly for seven weeks with formalized vaccines of 20^{hrs} serum-broth cultures).

Strain	Immune serum		
	Anti-961	Anti-1082	Anti-1088
961 original	1:256	1:128	1:128
961 "adapted"	1:8	1:8	1:4
1082 original	1:32	1:256	1:128
1082 "adapted"	1:8	1:16	1:4
1088 original	Spontaneous agglutination		
1088 "adapted"			
	1:8	1:8	1:4

1) The author is greatly indebted to Doctor Erna Moreh, Copenhagen, for this analysis, and for her careful serological assay of the behaviour of three of these strains in homologous immune sera as well as in 73 pneumococcal typing sera.

2) For these tests the author's thanks are due to Dr. K. Skadhauge, Copenhagen.

tween these strains, in that they were able to deplete each others' antisera of their agglutinins.

The three original strains, 961, 1082 and 1088 were tested with 73 pneumococcal typing sera: only 961 gave a capsular swelling reaction and agglutination, viz. in type 10 A serum. After "adaptation" to a diffuse growth the strain did not react any more in this serum but in type 12 serum, and there only by agglutination (1 : 32), the capsular swelling phenomenon being quite indefinite. On the other hand, the strain 1088 which had not reacted initially, did so after "adaptation", i. e. in the type sera 15, 15 A and 15 B by capsular swelling (titres up to 1 : 32) as well as agglutination (titres up to 1 : 128). Serum 15 C gave only a weak agglutination, and no capsular swelling.

The 961 rabbit immune serum reacted with both type 10 A and type 39 pneumococci, giving a capsular-swelling-titre of 1 : 8, and an agglutination titre of 1 : 16. The two other immune sera did not react with any of the 73 pneumococcal type strains tested.

Serum 961 did not react with either of two R pneumococcus strains tested, whereas sera prepared against strains 1082 and 1088 gave agglutinin titres up to 1 : 128 with these antigens. None of three R pneumococcal antisera did react with any of the three "adapted" strains.

Soluble Haemolysin.

Biological and physical characteristics.

The lysin production of recently isolated strains at times varied greatly, occasionally dropping even to zero. However, sufficiently frequent subcultivation of the strains made their lysin production rather stable. Rabbit and sheep blood corpuscles were haemolyzed to practically the same extent.

The lysin was thermolabile being partially inactivated when heated to 45° C. for 30 minutes, and completely destroyed by raising the temperature to 56° C. When stored at — 25° C. the lysin broth retained its activity unchanged for at least one month (Table 2). After prolonged storage the titre slowly decreased.

As a rule Seitz filtration reduced the lysin titre to some extent.

Table 2 also illustrates two other important properties of the lysin: (a) The lytic activity of the broth culture showed a considerable, 4 to 8-fold, in some instances even 16-fold increase after the first freezing. Such a rise in lysin titre was noted also after storage at + 4° C. (b) A proportion of the storage-inactivated lysin could be reactivated by treatment with some reducing agent, e. g. $\text{Na}_2\text{S}_2\text{O}_4$.

In these respects the lysin greatly resembles the pneumolysin (2). Both differ from the reversibly oxidizable streptolysin O in being somewhat more resistant to oxidation, at any rate to oxidation by hydrogen peroxide (Table 3).

Table 2.

The haemolytic activity of a 20^{hs} glucose-broth culture of strain 961 upon sheep red cells after varying periods of storage at $\pm 25^{\circ}$ C.

Days of storage at -25° C.	50 per cent haemolysis dosis
	ml.
0	0.016
1	0.002
7	0.002
29	0.008
40	0.032
53	0.032
58	0.064
58, and subsequent reduction with $\text{Na}_2\text{S}_2\text{O}_4$	0.008

Serological behaviour of the lysin.

The question arises of whether there exists any serological relationship between this haemolysin and the streptolysin O. A series of absorption experiments with homologous and heterologous antisera have been carried out to elucidate this point.

Technique. — Serum was diluted 1 : 50 with undiluted, active lysin broth. The mixture was incubated for 15 minutes in a water-bath at 37° C. and subsequently heated for 30 minutes at 56° C. The absorption was determined by routine antistreptolysin titration (AST) of the inactivated mixture. The technique used was principally that described by Ipsen (12) only slightly modified by the present writer (20). Control absorptions with heat-inactivated lysin broth or sterile broth showed no appreciable reduction of the serum titres.

Typical records of some absorption experiments are presented in Tables 4—6. Serum 6 from a rabbit immunized with the lysin-forming

Table 3.

The effect of oxidation and subsequent reduction on the haemolytic activity of various lysins.

Lysin	50 per cent haemolysis dosis			
	untreated	heated for 4 ^{hs} at 37° C.	treated with H_2O_2^1 for 4 ^{hs} at 37° C. without subsequent reduction	subsequently reduced with $\text{Na}_2\text{S}_2\text{O}_4^2$
Streptolysin O	0.008	0.032	1.0	0.008
Lysin 961	0.016	0.125	0.5	0.016
Pneumolysin	0.008	0.032	0.125	0.008

¹⁾ with 0.0375 per cent H_2O_2

²⁾ with 0.025 per cent $\text{Na}_2\text{S}_2\text{O}_4$

Table 4.

The effect of absorption with various lysins upon the anti-
"961"-lysin titre in rabbit immune serum.

Rabbit immune serum 6	Anti-,961"-lysin titre	
Before absorption		1:140
After absorption with	sterile broth	1:280
	lysins "961"	
	Seitz filtrate	\leq 1:36
	lysins "961"	
	broth culture	\leq 1:36
	streptolysin O	1:280

green-producing strain 961 was completely depleted of antilysin after absorption with unfiltered broth cultures as well as Seitz filtrates of the homologous strain (Table 4). A similar effect was achieved with the other green-producing strains tested, for example 1082. On the other hand, absorption with streptolysin O had no more effect than absorption with sterile broth.

The ordinary streptolysin-O-antibody in human sera is easily absorbed homologous streptolysin O (19), but not at all by the lysin formed by the green-producing strains investigated in the present study (Table 5). As will be shown later on, many patient sera neutralize the latter lysin more or less completely. The antilysin responsible for this neutralization was in some sera, for example B 1691 in Table 6, absorbed only by antigen of the homologous type, e. g. lysin "961", the heterologous streptolysin O proving inefficacious. In other sera a definite absorption even with heterologous O-lysin was noted, as recorded in Table 6 for sera B 1715 and B 1677. In serum B 1677 the heterologous absorption actually surpassed in effect that obtained with the homologous lysin.

The presence of lysin-forming green-producing streptococci in the throat prompts the question of whether specific antilysin is formed by

Table 5.

The effect of absorption with various lysins upon the anti-streptolysin-O-titre (AST) of patient sera.

Serum		A S T		
		B 1677	B 1691	B 1715
Before adsorption		1:560	1:800	1:1100
After absorption with	sterile broth	1:560	1:800	1:1100
	streptolysin O	\leq 1:36	\leq 1:36	\leq 1:36
	lysins "961"	1:560	1:1100	1:560

Table 6.

The effect of absorption with various lysins upon the anti-"961"-lysin titre of patient sera.

Serum	Anti-"961"-lysin titre		
	B 1677	B 1691	B 1715
Before absorption	1:140	1:560	1:400
After absorption with			
{ sterile broth	1:140	1:560	1:400
{ streptolysin O	\leq 1:36	1:400	1:140
{ lysin "961"	1:70	1:50	1:140

the carrier organism. If so, does this antilysin — in the following called "anti- α -streptolysin" — interfere with the ordinary antistreptolysin-O-reaction?

A number of patient sera have been simultaneously titrated with streptolysin O and lysin of green-producing coeci, as well as in some instances with pneumococcal lysin. There was considerable overlapping inhibition of the heterologous lysins by Todd's standard anti-streptolysin-O-immune serum. This made it possible to use the Todd immune serum as an arbitrary standard also for the other lysins, no homologous standard sera being available.

Figs. 4 and 5 summarize the titration results in patients' sera. The throat swabs of those 31 subjects, the serum titres of whom are given in Fig. 4 had been examined with particular regard to the pre-

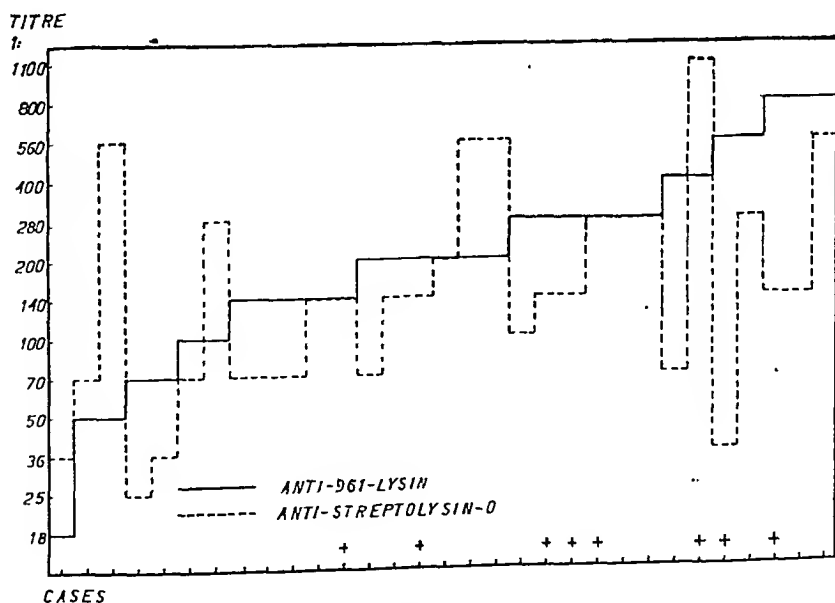


Fig. 4.

Patient sera titrated with streptolysin O and lysin 961 respectively.
+ soluble-lysin-forming green-producing streptococci found in throat swab.

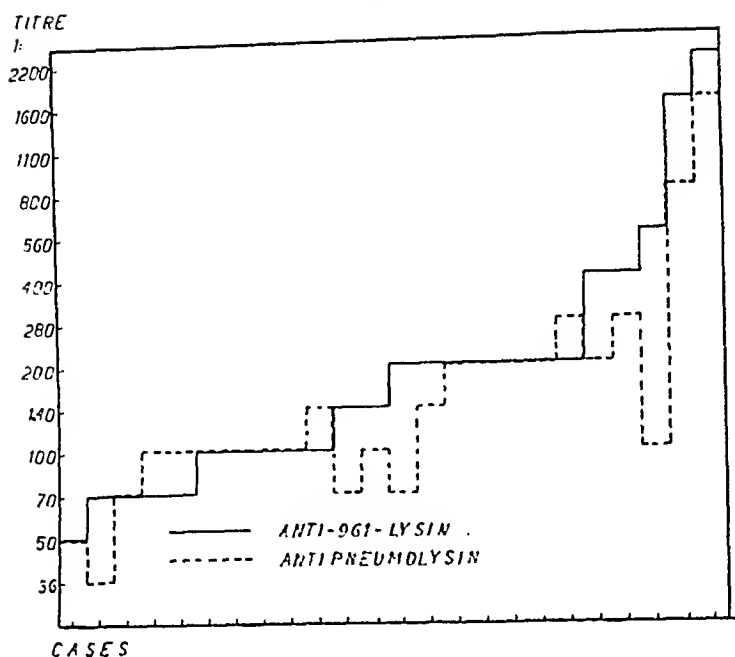


Fig. 5.

Patient sera titrated with lysin 961 and pneumolysin respectively.

sence of lysin-forming green-producing streptococci. Eight of them gave positive findings of such organisms (in the figure they are marked with a plus sign). Of 13 patients the anti- α -streptolysin titres of whom exceeded the arbitrary (Todd-serum-calibrated) border value of 200 units six, i. e. 46 %, had such streptococci, as against two or 11 % of 18 patients with lower titres. In other words, there seemed to ensue an actual stimulation of specific antibody formation.

From Fig. 5 it will be seen that a definite parallelism exists between the anti- α -streptolysin and anti-pneumolysin titres of the sera.

On the other hand, Fig. 4 suggests a certain connection to exist between the anti- α -streptolysin and anti-streptolysin-O-titres, in that sera with elevated antistreptolysin-O-titres (AST over 200 units) on an average also have a higher anti- α -streptolysin titre than sera with non-elevated AST. The mean anti- α -streptolysin titres were 200 and 64 units, for respective groups. However, considerable exceptions from the general trend were noted.

It seemed, thus of importance more in detail to analyse whether the presence of soluble-lysin-forming green-producing streptococci in the throat interferes with the AST of the patients. In Fig. 6 the AST frequency distribution among 27 β -streptococcus-negative patients found to harbour at least on some occasion, lysin-producing green streptococci, are compared with the corresponding distribution among 158 other β -streptococcus-negative patients collected at random and not examined for soluble-lysin-producing streptococci. There was no ten-

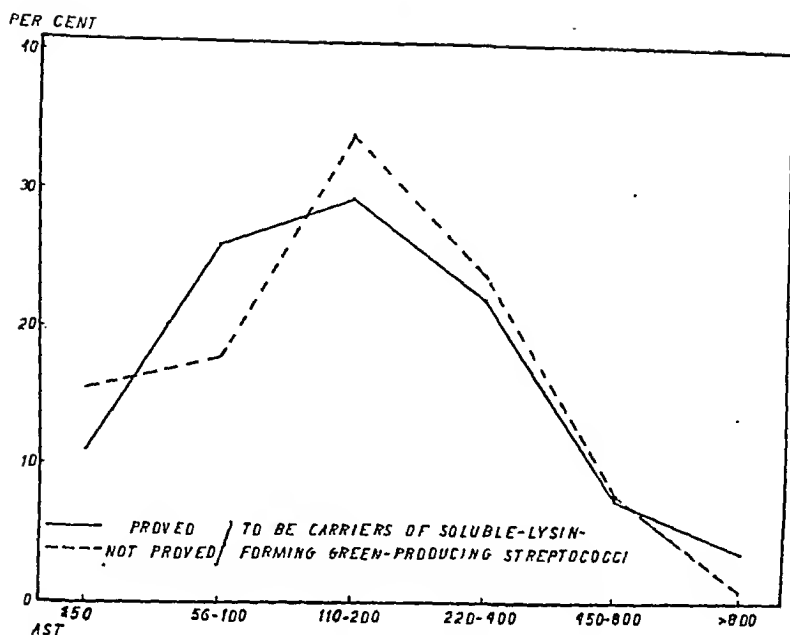


Fig. 6.

Frequency distribution of the antistreptolysin titres (AST) among 27 β -streptococcus-negative carriers of soluble-lysin-forming green-producing streptococci, and 158 patients collected at random, similarly without β -streptococci.

deney towards raised AST values in the former group. Thirty-three per cent of them had an AST over 200 units as against 32 per cent of the other group of β -streptococcus-negative subjects.

Discussion.

As early as in 1934 Todd (24) reported that high-titre anti-streptolysin-O sera from hyperimmunized horses neutralized also pneumococcus haemolysin, though more antibody was required than for neutralizing streptolysin O. Nevertheless, no distinct rise of the AST in (human) sera from cases of clinical pneumococcal infections has been observed (26). On the other hand, pneumococcal infections do raise the antipneumolysin titre (17, 18, 24).

The great resemblance between the soluble lysin discussed in the present study and the pneumococcal lysin, as well as between their specific antibodies, is remarkable. Their possible identity should be seriously considered. This makes the interest gravitate towards the much debated problem of the differentiation of R pneumococci from alpha-streptococci, and the transformation of one into the other. This problem cannot, however, be fully discussed here. Several surveys of the subject have been published (11, 16). Opinions are still very controversial.

Furthermore, attention should also be called to observations indicating the existence of β -streptococci, which under certain condi-

tions produce a definite α -haemolysis in blood agar plates (2, 4, 9, 10). However, closer scrutiny of the data reported reveals the non-identity of these strains and those dealt with in the present paper.

Recently Moreh (13, 14) has reported on the existence of encapsulated streptococci serologically related to certain types of pneumococci. She isolated from human throats two α -streptococcal strains possessing capsules which gave a distinct swelling reaction in certain pneumococcal typing sera. They were completely insoluble in bile, resistant to optochin 1 : 50,000, and one of them reacted with Lancefield's group H serum.

As early as in 1934, Paul (23) stated that "a growing literature bears witness to the fact that the actual differences between some R pneumococci and streptococci are occasionally so slight as to reach the vanishing point". All or most of the customary diagnostic characteristics, viz. capsules containing specific polysaccharide, diffuse growth in fluid media, bile solubility, optochin sensitivity, mouse pathogenicity, etc. are more or less completely lost when the S pneumococci dissociate to R forms. This occurs with old laboratory strains, or when the pneumococcus is grown in the presence of specific immune serum, bile, optochin, or similar agents. Such variants may be very hard to differentiate from alpha-streptococci. According to Paul (23), their reversibility to the parent S type is the ultimate criterion upon which ought to rest the claim that they actually are pneumococci. However, the fulfilment of this postulate seems to be attainable only in a few instances (23). On the other hand, the suppression of the solubility in bile is rarely complete. Most R strains of pneumococci display only an increased resistance to the action of bile — as did the strains studied here — while very few become really insoluble in bile (8, 22).

Wilson and Miles (25) state: "It has been quite clearly demonstrated within recent years (3, 5, 11) that the pneumococcus, when grown under suitable conditions, produces a soluble haemolysin of the oxygen-sensitive, heat-sensitive type, which undergoes reversible oxidation at low temperatures. Whether other streptococci that produce α -haemolysis would also elaborate a filtrable haemolysin under suitable conditions is at present unknown." Of course, such a statement seems to support the supposed pneumococcal nature of the streptococcal strains discussed in this paper.

As a matter of fact, they show a great resemblance to the extraordinarily rough R pneumococci described by Dawson (6, 7) as dissociants developing from ordinary R pneumococci when subjected to prolonged cultivation at 37° C. He thought that they represented the "true" R pneumococci, whereas the form previously regarded as R might be better termed S, and the S form in turn as M (mucoid) (7). Dawson's "true" R pneumococci consisted of plump cocci and coccobacilli in tangled masses of enormous chains and skeins. In fluid media

they gave a sedimented type of growth resembling a tuft of cotton at the bottom of the tube. They were soluble in bile, and possessed comparatively little virulence for mice. No statements were made as to their haemolysin production, capsule formation, or serological behaviour.

It is of course possible that a dissociation of ordinary pneumococci into Dawson's ultimate "true R phase" occurs not only under experimental conditions, but also in a "natural" environment such as the human throat. But if this is true, it is to be admitted that the persistency of capsules about such pneumococcal dissociants is a rather unexpected phenomenon. Anyhow, Dawson (6) has actually reported that colonies similar to his extremely rough variant were frequently found in cultures from normal throats. Furthermore, in a study of 40 rough methaemoglobin-producing colonies in sputum cultures from subjects with respiratory infections, Paul (21) found 12 bile-soluble strains. Two of them consistently exhibited reactions which were typical of R pneumococci. The other ten did not offer definite evidence of being R pneumococci, but it seemed not unlikely that they represented intermediate or related forms.

The existence in the normal human throat flora of R pneumococci more or less capable of reversion into virulent S forms may constitute an important reservoir for the ubiquitous pneumococcal agent.

Summary.

From the human throat have been isolated eneapsulated green-producing streptococci which formed a soluble haemolysin. They seemed to be closely related to, or even identical with, Dawson's "true R phase" pneumococci. They grew in extremely rough colonies, and gave in fluid media a sedimented type of growth resembling a tuft of cotton. They were only moderately sensitive to bile and optochin. There was a definite serological relationship between several of these green-producing strains and various pneumococcal types.

The soluble haemolysin formed by these strains was found to be closely related to the pneumococcal lysin. On the other hand, the relationship to streptolysin O was far less close, although some serological overlapping did occur. However, no definite interference with the antistreptolysin reaction could be demonstrated. Human carriers of such soluble-haemolysin-producing micro-organisms were found to some extent to form specific antilysin.

The observations lead up to a discussion of the taxonomy of pneumococci and alpha-streptococci.

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POSITION DANS LA SYSTEMATIQUE ET NOMENCLATURE D'*HAVERHILLIA MONILIFORMIS*

Par A. R. Prévot.

(Reçu par la redaction février le 9e 1948.)

Dans un article récent, Thjötta et Jonsen (1), ayant étudié une souche de *H.moniliformis*, ont proposé de la nommer *Streptothrix muris ratti* Schottmuller 1914. J'ai moi-même proposé en 1946 (2) une autre combinaison: *Haverhillia moniliformis* (L. N. P.) nv. comb. et me propose dans cette note de soutenir cette appellation.

1. *Genre*. Le terme générique *Streptothrix* proposé par Cohn en 1875, n'a jamais été défini et est actuellement, de ce fait, tombé en désuétude, remplacé par *Actinomyces*, Harz, bien défini par la morphologie de son espèce-type *A. bovis*: »filament à extrémités renflées, mycélium multiramifié, divisé en segments fonctionnant comme conidies, immobile, Gram-positif«. Thjötta et Jonsen ayant eux-même rejeté le rattachement au genre *Actinomyces* dont l'espèce *moniliformis* n'a pas les caractères (absence ou ébauche de ramifications, Gram-négative) on comprend mal pourquoi ils le rattachent à *Streptothrix*, synonyme actuellement inusité.

Quand on examine l'ensemble des genres bien définis et admis, on n'en voit aucun pouvant admettre l'espèce *moniliformis* sauf le genre *Haverhillia*, proposé par Parker et Hudson en 1926 (3) et défini: »bâtonnets et filaments Gram-négatifs, polymorphes, formes renflées et sphéroïdes, immobiles, ébauche de ramification; besoin de facteur X et V, anaérobie facultatif«. Ce genre est correctement formé et en accord avec le Code international de Nomenclature, terminé à Copenhague en 1947. J'ai précisé (4) sa position dans la systématique en 1946: il se rapproche morphologiquement du genre *Spherophorus* dont il ne se distingue que par l'anaérobiose facultative et le besoin en facteurs X et V. Je le considère donc comme un sous-genre de

1) Thjötta et Jonsen. Acta Path. et Microb. Scand., 1947. XXIV. 334.

2) A. R. Prévot. Ann. Inst. Pasteur 1946. 72. 297.

3) Parker et Hudson. Am. Journ. of Path., 1926. II. 357.

Spherophorus et ainsi, le place dans la famille de *Spherophoraceae* (4) ou Actinomyceetales Gram-négatives.

II. *Espèce*: Le premier nom spécifique correctement défini et correspondant à une espèce bien décrite est incontestablement *moniliformis* Levaditi, Nicolau et Poineloux 1925. Le nom spécifique *multiformis* est plus récent d'un an (5). Le nom de l'espèce actuellement en cause est donc *Haverhillia moniliformis* (L. N. P.) P. 1946.

Cette espèce est-elle identique à celle qui a été décrite en 1914 par Schottmuller? Il est difficile de l'affirmer, la souche de Schottmuller étant disparue et n'ayant pas été suffisamment étudiée, la confrontation est impossible. Il est également difficile d'affirmer l'identité de *H. moniliformis* avec l'espèce *H. piliformis* Tizzer 1917—18, qui est certainement très voisine mais non totalement la même. A ce sujet il est bon de se reporter à la multiplicité des espèces du genre voisin *Spherophorus*, espèce-type. *Sph. funduliformis* qui comprend 17 autres espèces très voisines mais toutes très bien individualisées et dont l'autonomie s'affirme chaque jour davantage (4). Il est probable que le genre *Haverhillia* renferme, comme tous les genres pathogènes pour l'homme et les animaux des espèces nombreuses et très voisines, dont l'espèce-type serait *H. moniliformis* (Syn: *H. multiformis*) et les autres espèces: *H. muris rattii*, *H. piliformis*, en tout très voisines, mais pas forcément identiques. Aussi, quand une souche de ce genre est isolée, il y aurait intérêt à déterminer tous ses caractères non seulement morphologiques et culturels, mais encore sa structure antigénique, sa biochimie, afin de savoir si cet hôte de la cavité buccale de nombreux rongeurs: souris, rats, rats-palmistes, etc., est unique ou multiple.

4) A. R. Prévot. Ann. Inst. Pasteur, 1938, 60, 280, et Manuel de Classification des Anaérobies, 2e Edition, Masson, Paris 1948, p. 226.

5) Levaditi, Nicolau et Poineloux. C. R. Acad. Sc., 1925, 180, 1188.

A SHORT REPLY TO A. R. PRÉVOT

By Th. Thjötta and Jon Jonsen.

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When we preferred the name *Streptothrix muris ratti* for the microbe in question the reason is that this is the name given the microbe by Schottmüller in his paper of 1914, when this organism was first described. It does not seem doubtfull that Schottmüller really had the organism in his hands at that time and thus had the priority of the name. Besides, the term *Streptothrix* gives some impression of the microbe as being a more or less threadlike and chainlike organism, while the term *Haverhillia* does not give any impression whatever of any character, but merely alludes to a small town in Massachusetts, unknown to the majority of mankind. Further the definition of *Haverhillia* does not very well meet the characters of the microbe described by us, as our strain did not show any branching and was not dependent upon X and V.

However, the most important question in this connection is the avoidance of the term *Actinomyces* and the reaching of a name understood and used by all. On that ground we are quite willing to second Dr. Prevots proposal and accept his term *Haverhillia moniliformis*, especially as the speciesname *moniliformis* gives a very good impression of one of the morphological factures of this organism.

THE ROLE OF THEBESIAN DRAINAGE IN THE DYNAMICS OF CORONARY FLOW IN CASES WITH AND WITHOUT CORONARY SCLEROSIS

By Pentti I. Halonen and Alvari Aho.

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As early as 1898 *Pratt* suggested that the cardiac muscle might be fed by some accessory channels apart from the coronary arteries. Strong support for this view has been received from cases with obliteration of long standing of both coronary arteries during life. Such cases have been described by *Pratt* (1898), *Kretz* (1928), *Leary* and *Wearn* (1930), *Cabot* and *Mallory* (1930), and by *Scott* and *Holz* (1931). Cases in which the narrowing of the coronary arteries is out of proportion to the data of the clinical history, are also surprisingly frequent on autopsy boards. This fact forces us to seek for some other channels, apart from the coronary arteries, which might have served as portals of entry for the nourishing blood into the heart muscle. According to *Bellet* (1946) the following ways may be considered: 1) extracardiac anastomoses, 2) the Thebesian vessels, 3) reversal of flow in the coronary veins. *Bellet* points out, basing his view mainly on the investigations of *Langer* (1880), that extracardiac anastomoses, owing to their small size and number, and their location along the bases of the aorta and the pulmonary artery, cannot be of major importance clinically. It is quite possible, on the other hand, concludes *Bellet*, that the Thebesian veins by virtue of their number may contribute to the feeding of the heart muscle all the more so as the narrowing of the coronary arteries favours a reversal flow in them. *Poirier*, *Charpy* and *Nicoles* (1912), and also *Yater* (1926), it is true, demonstrated the fact that, in the presence of complete occlusion of both coronary arteries, blood may be transmitted by auricular contraction from veins into capillaries, and conversely, from capillaries

into auricles by ventricular contraction. In *Bellet*' opinion it is of less significance to the nutrition of the heart muscle, this being venous blood and poor in oxygen.

The number of the Thebesian channels and their distribution between the various sections of the heart has been subject to numerous investigations (e. g. *Kretz* 1928, *Wearn* 1928, 1933, *Grant* and *Viko* 1930, *Ungern* 1934, 1938, *Halonen* and *Tossavainen* 1941). In this study we are making an attempt to determine the number of the Thebesian channels in the various sections of the heart. In doing that, we have devoted particular attention to observing those cases in which an advanced coronary sclerosis is present, in order to find out whether there be some coincidence of changes in the total volume of the Thebesian channels, either in the whole of the heart or eventually in the ventricles alone, where the demand for food is greatest.

The method we have employed is very much the same as that used by *Lendrum*, *Kondo* and *Katz* (1945) in investigating in dogs the role of the Thebesian channels and the coronary veins in the dynamics of the blood flow in the various sections of the heart. We were, however, not aware of their investigations beforehand. The procedure used by us was, briefly, the following: The hearts of the test animals (calves) were removed immediately after they were sacrificed. Also the hearts of the human subjects were, as a rule, taken immediately after death. As a matter of fact, hearts removed later than 24 hours after death were never used. The auriculo-ventricular orifices were sealed with an artificial valve device, consisting of a rubber ring covered with a rubber membrane, which was by means of »kochers« and the fingers introduced through the aorta or the pulmonary artery into the corresponding cardiac cavities and placed in the A-V openings. The next step was to pass a drainage cannula through the superior vena cava into the coronary sinus and another one by the same route directly into the right auricle. The venae cavae were, both of them, fastened with a ligature tightly to the cannulae. One of the pulmonary veins of the left auricle was cannulated, the others were completely occluded. Drainage cannulae were then inserted through the aorta into each of the two coronary arteries and a third one into the left ventricle. One of the cannulae was passed by way of the pulmonary artery into the right ventricle. The aorta and the pulmonary artery were then tightened round the cannulae. The next step was to rest the artificial A-V valves for tightness by injecting physiological NaCl solution into the two auricles and ventricles of the heart at a slight extra pressure. When observing the cavities beyond the devices after the injection, the absence of leakage was proved to be complete. Finally, both coronary arteries were perfused with physiological saline solution at a constant pressure of 150 mm Hg, and after the lapse of a fixed period of time the various drainage components were calculated, i. e. the flow into the coronary sinus, the right auricle and ventricle, the left auricle and

ventricle, was measured. The initial pressure ranged from 100 to 200 mm Hg, but since no appreciable difference could be observed between the amounts of fluid drained through the different channels, a constant pressure of 150 mm Hg was maintained during the later stages of the procedure. The method used by us differs from that of *Lendrum* and collaborators in two respects: 1) their artificial A-V partition is unlike our rubber ring device, 2) they collected the fluid drained into the left ventricle by means of a cannula introduced into it through a stab wound at the apex.

We understand that the described method cannot yield any absolute values. The cardiac oedema, which develops during the course of the experiment, and which constitutes an increasing load on the myocardium and its small vessels, already eliminates the possibility of obtaining such values. In order to secure more accuracy we have endeavoured to perform all the experiments under identical conditions. Moreover, no guarantee can be given that a slight leakage does not take place past the margins of the artificial A-V valves. Nor can the collection and measurement of the fluid volumes be absolutely accurate. All the experiments showed, in addition, tissue leakage, the amount of which varied, but never exceeded 13 per cent, as the following tables will demonstrate. In spite of the possibility of considerable inaccuracies, — maybe even others than those mentioned —, we nevertheless hold that some picture of the volume of the Thebesian channels in the various sections of the heart and of possible larger variations in that volume, may be obtained by means of the method describes. The below table summarizes the data yielded by this method in our experiments on calves' hearts.

Table 1.
Partition of coronary flow drainage in calf.

No.	Coronary sinus	Right auricle	Right ventricle	Left auricle	Left ventricle	Right heart	Left heart	Tissue leakage
1	35	7	30	3	20	72	23	5
2	42	6	25	2	19	73	21	6
3	31	12	35	4	10	78	14	8
4	40	2	31	1	16	73	17	10
5	34	7	39	5	8	80	13	7
6	45	3	30	2	20	78	22	—
7	29	20	34	2	10	83	12	5
8	30	24	23	4	12	77	16	7
9	37	5	38	1	10	80	11	9
10	40	4	36	5	9	80	14	6
Mean	36,3	9,0	32,1	2,9	13,4	77,4	16,3	6,3
Standard deviation	36,3±1,1	9,0±1,6	32,1±3,4	2,9±1,3	13,4±2,6	77,4±2,5	16±2,6	6,3±1,7

It appears from this table that considerable divergencies between the various sections of the heart can be demonstrated in various subjects. It is striking, too, that a substantial part of the fluid drained via the Thebesian channels empties itself into the ventricles. The distribution of the right heart drainage between the coronary sinus and the right ventricle is fairly equal, the amounts of the fluid drained directly into the right auricle, on the other hand, being considerably smaller. These figures are very much the same as the values obtained in dogs by *Lendrum*, *Kondo* and *Katz*, with the exception of the right auricle drainage value, which is appreciably higher in their findings. It is of interest to note that the values of the left and right heart coronary flow drainages obtained by *Lendrum* and his collaborators by means of this method correspond to the coronary flow drainage values in the experiments of *Katz*, *Jochim* and *Weinstein* (1938) yielded by live hearts.

In order to find out whether there be some evidence of an increase in the volume of the Thebesian channels in man in the presence of coronary sclerosis, as might be expected, provided they act as substituting factors, we have employed the method described above in investigating 16 human hearts, which did not show any signs of coronary sclerosis and 13 human hearts with considerable coronary sclerosis. The results appear in the following tables (No 2 and 3).

Table 2.

Partition of coronary flow drainage in man without coronary sclerosis.

No.	Coronary sinus	Right auricle	Right ventricle	Left auricle	Left ventricle	Right heart	Left heart	Tissue leakage
1	17	5	21	6	41	43	47	10
2	20	7	30	3	30	57	33	10
3	25	7	33	3	22	65	25	10
4	40	10	23	2	15	73	17	10
5	20	7	28	10	22	55	32	13
6	29	7	28	10	21	64	31	5
7	24	6	32	3	24	62	27	11
8	24	10	26	1	30	60	31	9
9	41	4	25	2	18	70	20	10
10	27	5	20	2	36	52	38	10
11	21	8	30	4	29	59	33	8
12	25	6	28	3	27	59	30	11
13	42	5	25	1	20	72	21	7
14	32	8	35	5	20	75	25	—
15	25	5	32	4	24	62	28	10
16	46	5	16	3	20	67	23	10
Mean	28,6	6,5	27,0	3,9	24,9	62	29,3	9,0
Standard deviation	28,6±2,4	6,5±0,6	27,0±4,2	3,9±2,2	24,9±1,8	62,0±2,7	29,3±4,4	9,0±0,8

Table 3.

Partition of coronary flow drainage in man in cases of coronary sclerosis.

No.	Coronary sinus	Right auricle	Right ventricle	Left auricle	Left ventricle	Right heart	Left heart	Tissue leakage
1	32	6	20	4	34	58	38	4
2	45	4	20	2	21	69	23	8
3	53	6	25	2	14	84	26	—
4	40	14	20	4	15	74	19	7
5	44	5	27	5	19	76	24	—
6	48	10	22	2	9	80	11	9
7	30	9	39	2	10	78	12	10
8	37	13	35	1	9	85	10	5
9	40	12	23	3	12	75	15	10
10	25	17	33	2	12	75	14	11
11	27	22	30	2	10	79	12	9
12	46	5	21	1	20	72	21	7
13	40	8	23	3	17	71	20	9
Mean	39	10,1	26,0	2,5	15,5	75,1	18	6,2
Standard deviation	39,0±2,0	10,1±1,3	26,0±1,5	2,5±0,3	15,5±1,2	75,1±1,7	18±1,9	6,2±0,6

On closer examination of table 2 we observe that the rôle of the Thebesian channels in the drainage of the ventricles is of far greater importance than earlier investigations of injected preparations have been able to demonstrate. In addition to this great individual variation can be noted. The rôle of the Thebesian channels appear to be significant in the venous circuit of the right ventricle. In the right auricle they are obviously of less importance, the fact being that several other veins empty themselves into the right auricle apart from the coronary sinus, namely vena cordis parva, venae ventrales, vena Galeni and the s. c. Zuckerkandl's channel. According to *Gregg, Shipley and Bidder* (1943) a large amount of blood is drained in vivo, at least via venae ventrales, directly into the right auricle.

If we compare the coronary drainage values in cases with coronary sclerosis with the values of healthy hearts, no appreciable differences can be noted. Not even the ventricles show, as might be expected, larger coronary drainage values in the presence of coronary sclerosis.

In short, we have not been able to demonstrate in instances of coronary sclerosis and 13 human hearts with considerable coronary channels. This does not mean, however, that a reverse feeding of the heart muscle from the direction of the cardiac cavities by way of the Thebesian channels cannot take place in those cases in which coronary sclerosis is present. It is well known that the bases of the coronary vessels, in case a narrowing process in them occurs, show a reduced pressure, and this conditions facilitates the reverse flow from the cardiac cavities in the direction of the periphery via the Thebesian channels.

Summary.

The coronary arteries of hearts were perfused with physiological saline immediately after sacrificing the animals, and the coronary arteries of human hearts in the same manner soon after death, both in case which presented a very marked coronary sclerosis and in those in which no coronary sclerosis could be shown. The volumes of the drainage into the coronary sinus, the right auricle and ventricle, the left auricle and ventricle were measured separately. Special artificial partitions were used to seal the auriculo-ventricular openings.

The technical details and the inaccuracies of the method have been described.

The detailed results have been presented in tables. The flow into the right ventricle via the Thebesian channels was shown to be significant magnitude both in human and in calf hearts.

The Thebesian drainage was not more significant in cases with coronary sclerosis than in those without any sclerotic condition.

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A CASE OF WEGENER'S GRANULOMATOSIS

By *Sven Johnsson.*

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Generalized vascular diseases are of great interest both from an etiological, pathological-anatomical, and clinical point of view. Foremost among these are periarteritis nodosa, rheumatic arteritis and thrombo-angiitis obliterans, but the generalized vascular changes which are sometimes observed in septic conditions are also of definite interest.

Wegener (1936, 1939) was the first to describe as a special disease a both clinically and pathologically-anatomically well characterized condition with vascular changes similar to periarteritis nodosa and with formation of granulomas in several organs.

Clinically the disease is characterized by a fatal outcome after 4—7 months; it begins with rhinitis, developing into a gangrenous-neerotic process of the nose, throat and respiratory tract with a septic picture, high sedimentation rate and, finally, uremia without elevated blood pressure.

The pathological-anatomical examination reveals an ulcerous-neerotic inflammation within the nasal cavity, throat, and larynx as well as generalized vascular changes. Microscopically most organs show pictures suggestive of periarteritis nodosa. In the kidneys, moreover, numerous periglomerular granulomas are seen. These consist of radially arranged fibroblasts with leukocytes, lymphocytes, and plasma cells. The glomeruli within the granulomas are entirely or partly necrotic, sometimes replaced by fibrous tissue.

After *Wegener* (three cases) five identical cases have been published (*Postel & Laas*, 1941, 2 cases; *Lindsay et al.*, 1944, one case, and *Ringertz*, 1947, two cases). These 8 cases all showed changes in the lungs and they had developed renal failure with uremia. The changes were most pronounced in the lungs, respiratory tract, spleen, and kidneys. The most pronounced changes were the conspicuous periglomerular granulomas deviating from the ordinary picture of peri-

arteritis nodosa. The other organs showed arterial changes similar to periarteritis nodosa, as well as aneurysms, and, in the lungs, pneumonias with diffuse necroses and giant cells. The changes are to a certain degree similar to those found in allergic cases, where asthma or rheuma has dominated the morbid picture. All these authors have considered the disease to be related to periarteritis nodosa.

One more case will be reported in addition to these 8 cases which, moreover, presents a clinical picture illustrating a difficult differential diagnosis.

Case report:

Oto-rhin. lar. Journ. 528/43 B. S. ♀ 57 years old.

The patient has previously been in essentially good health. One or two months before admittance to the hospital she noticed that the hearing in the right ear was reduced. Examination of the ear 16.4.43 revealed an inflamed nasal mucous membrane and transudate in the right middle ear. Paracentesis was performed. Ear-ache and sparse secretion set in. The hearing gradually became more impaired and at the same time the patient experienced pains radiating from the right ear down towards the neck.

The patient was treated in the oto-laryngological clinic from 4.5—26.5 1943.

Status (on admittance): General condition somewhat affected. Temperature about 38°. Blood pressure 130/80. Right drum-membrane slightly inflamed and thick; paracentesis was made without result. Laboratory examination: Hemoglobin 80 %, erythrocytes 4,260,000. Leukocytes 8900; Neutrophils 76 %, lymphocytes 22 %, monocytes 2 %, eosinophiles 0. Sedimentation rate 72 mm. per hour.

Leukocytes (13.5) 12600. Non-protein nitrogen (14.5) 36 mg %. Albumin (14.5) negative. Urine (14.5) 8—10 leukocytes, 1—2 erythrocytes per field.

Since the patient's affected general condition and the temperature arising to around 39° could not be accounted for by the mild ear symptoms, the following examinations were carried out:

Chest radiogram: Multiple rounded parenchymal densities — suspected to be metastases (Fig. 1). *Plain radiogram* (kidneys): Upper left kidney changed into a nearly fist-sized rounded density. *Urography:* Similar contrast density bilaterally. Morphology of left renal pelvis normal. *Röntgen diagnosis:* A tumor in the left kidney with chest metastases.

Neither radiological or surgical therapy was considered to be indicated. The patient died 26.5. No urine examinations or non-protein nitrogen determinations were made after 14.5.

Clinical diagnosis: Nephromata of the left kidney with pulmonary metastases.

From the autopsy report: (215/43: C. G. Ablström).

Gross Examination.

Slight bilateral edema of the ankles. No palpable surface lymph nodes; no cutaneous bleedings. No tumour in the breasts.

The heart somewhat enlarged, with a soft consistency. Heart valves normal. Slight coronary sclerosis. No thrombi in the chambers of the heart or in the main stems of the pulmonary vessels.

Pharyngeal mucous membrane pale. No tonsillar enlargement. Thyroid gland normal.

Pleural cavities normal. Trachea and larger bronchi showed inflamed mucous membrane and a seropurulent exudate. All large and medium-sized bronchi were dissected but no tumor could be observed. In all the pulmonary lobes many rounded, rather soft, nodes were felt, varying in size from a walnut to a mandarine. On incision these nodes were revealed as greyish



Fig. 1.

Chest radiogram: Multiple rounded parenchymal densities.

yellow medullar necroses, often with central softening. Towards the periphery some necrotic foci continued as yellow reticular streaks into the surrounding lung tissue. Upon compression a cloudy liquid was expelled. Most foci were sharply defined from their surroundings, being somewhat elevated. Macroscopically many of them gave the impression of tumor-metastases (Fig. 2). Besides the palpated nodes numerous similar small nodules on the cut surface within the lower and medial lobes were observed, distributed diffusely within the pulmonary parenchyma. Hilar and mediastinal lymph nodes appeared unaltered.

Peritoneal cavity normal. Gastro-enteric tract presented no abnormalities. Liver showed only toxic influence. No bleedings and no macroscopic necroses were observed. Gallbladder and the biliary ducts were normal. Spleen was much enlarged, very flaccid, with a greyish red, very soft parenchyma leaving a large deposit on the knife. At the upper pole the spleen felt somewhat firmer and a greyish yellow necrosis with diffuse outlines was found, in appearance similar to the necrotic areas in the lungs. Pancreas normal without any sign of tumor.

Kidneys: In the upper pole of the left kidney there was a larger than mandarine-sized, unilocular, thin-walled cyst containing a clear liquid. The cyst had no communication with the renal pelvis. The kidneys were otherwise normal in size and form. The capsule could be easily stripped from the cortical surface of the kidney. The surface of both kidneys showed several light grey irregular areas, usually surrounded by hemorrhagic zones. The cut surface of the renal parenchyma showed these areas to be triangular or more irregularly formed anemic infarcts with the apex towards the centre. They were mainly within the cortex and were surrounded by the hemorrhagic zones described above (Fig. 3). The corresponding renal vessels were filled with thrombi. No tumor in the kidneys. The adrenal glands were of normal size with a cortex rather rich in lipoids and no sign of tumor. In the uterus some slightly larger than hazelnut-sized myomas were found as well as a small cervical polypus.

The cranium was not opened.



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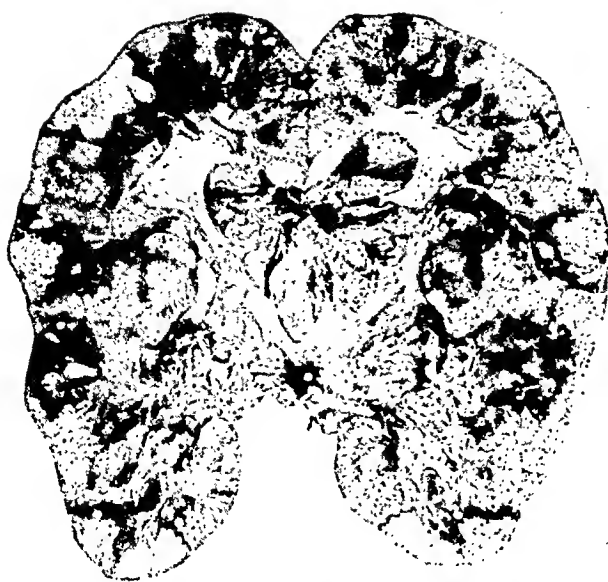
Fig. 2.

The cut surface of the right lung showing multiple, more or less well defined necrotic foci.

Microscopic Examination.

Lungs: The greyish yellow foci consisted of necrotic or necrobiotic lung tissue where the lung structure could be surmised. The cell nuclei were either entirely dissolved, fragmentary, or could be seen dimly, like shadows. Here and there within the necroses leukocytes were seen, often in a state of decomposition. The foci were surrounded by a wall of alveoli, filled with a fibrinous exsudate. Abundant leukocytes were seen within this wall and also some lymphocytes. These occurred not only within the alveoli but also in the alveolar walls, which sometimes showed a certain fibrous induration. Near the foci the capillaries were dilated and filled with erythrocytes; there were also minor bleedings in the pulmonary parenchyma. Farther away from the foci the fibrinous exsudation in the alveoli decreased and in the periphery the lung tissue was normal. The reticular streaks in the surrounding lung tissue were shown under the microscope to correspond to striated necroses, sometimes of fibrinoid character.

In addition to these necroses, changes in the vascular system were prominent features in the histological picture. They appeared most clearly within the medium-sized and larger vessels, both arterial and venous. The most outstanding and initial change was a thickening of the intima which



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Fig. 3.

Numerous anemic infarcts in the cortex of the right kidney.

reduced and sometimes obliterated the vascular lumen. The thickening of the intima was caused by an edematous swelling of the subendothelial tissue together with a proliferation of the subendothelial cells (Figs 4—5). The thickened intima also contained a few neutrophile leukocytes, lymphocytes, and plasma cells; sometimes also multinucleated giant cells with marginally located nuclei surrounding a pale protoplasmic zone (Fig. 6). Here and there the thickened intima showed foci of fibrinoid necroses. The thickening of the intima was sometimes more pronounced within one part of the circumference than within another and the lumen was then displaced eccentrically. The intimal endothelium was always well preserved. In several places the changes were not confined to the intima only: the entire vascular wall was necrotized and infiltrated with inflammatory cells. The elastic lamellae in the media were entirely or partially destroyed. Within many vessels changed in this manner a fibrinous thrombus, often interspersed by leukocytes, was found on the intima. The thromb could fill the lumen partially or completely: in the first case it often formed a small cushion- or knot-shaped exerescence on the intima. The thrombotic layers were as a rule of recent date, but in a few places they were organized and showed at times even an ineipient recanalization.

A dominating characteristic was that all these vascular changes occurred in the periphery of the pulmonary foci, which were thus surrounded by a ring of affected vessels. There was scarcely ever any vascular deterioration without a greater or lesser necrotic focus within the vicinity. The thickening of the intima was sometimes localized precisely in the sector of the vessels facing the necrotic lung focus, while the intima was quite normal within the other parts of the circumference.

Kidneys: The numerous large anemic infarcts which were triangular or quadrangular in form, situated mainly in the cortex, dominated the microscopic picture of the kidneys. The renal tissue showed here a more or less pronounced necrosis. In the periphery some small dilated hyperemic vessels

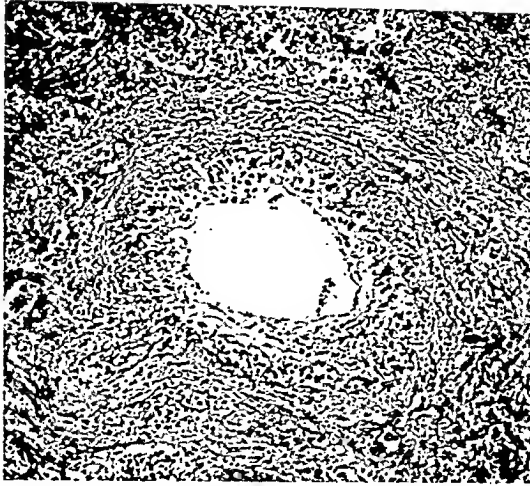


Fig. 4.

A pulmonary vessel with thickened intima and infiltrated by inflammatory cells.

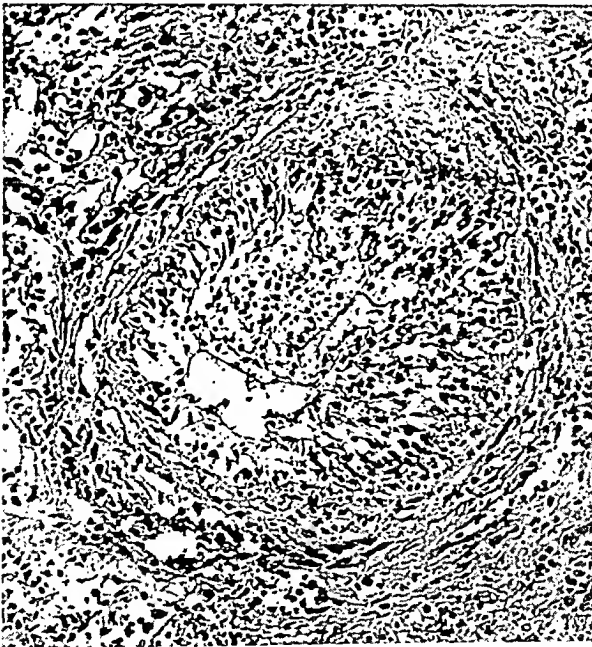


Fig. 5.

A pulmonary vessel with a so pronounced thickening of the intima that the lumen is almost completely obstructed. The intimal endothelium intact.



Fig. 6.

A pulmonary vessel with giant cells in the inflamed intima.

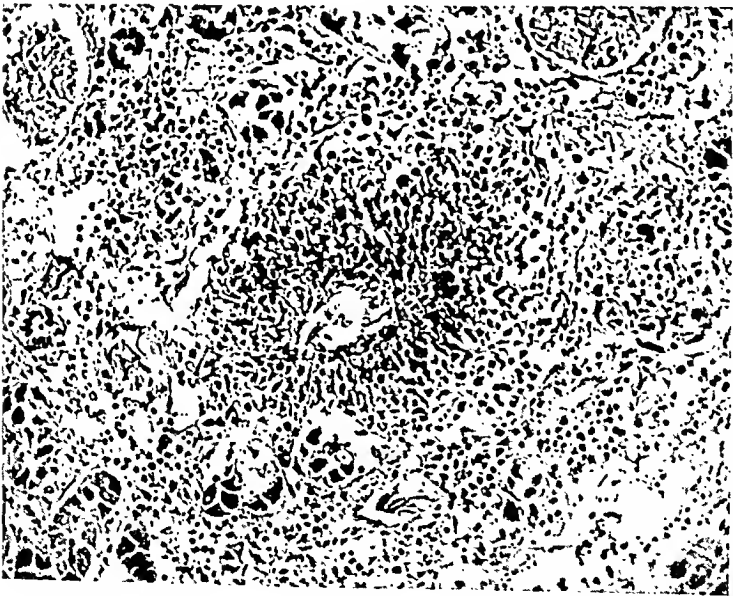


Fig. 7.

Perivascular granuloma of the kidney with fibrinoid necroses of the vessel wall.

were found with bleedings into the parenchyma. In large renal arteries connected with the infarcts the lumen was found to be filled with thrombi, which were composed of fibrin with a more or less ample infiltration of leukocytes. The vascular walls showed inflammatory changes of varying intensity in the form of an infiltration of leukocytes, in some places causing a complete destruction of the vascular wall (Fig. 7). In other places the wall changes were limited to the intima and consisted of an edematous thickening and sparse infiltration of leukocytes. Vascular changes of this type occurred also in non-thrombotic vessels. In principle they were of the same character as in the lungs. The larger venae near the infarcts could be the seat of thrombi, which, however, were of more recent date and appeared to be of secondary nature. There were also changes in the glomeruli. In their mildest form they occurred as a fibrinoid necrosis of one or a few loops, combined with a sparse infiltration of leukocytes. In advanced cases the entire glomerulus had been transformed into a small round granuloma, composed of histiocytes, fibroblasts, and a rather large amount of leukocytes (Fig. 8). There were numerous leuko- and lymphocytes also in the tissues in the vicinity of these transformed glomeruli.

The spleen showed anemic necroses and necrobiotic areas where only the cell nuclei were discerned. Several large vessels, probably both arteries and veins, showed necrotic vascular changes, sometimes of fibrinoid character. Numerous neutrophile leukocytes were found in and around the destroyed vessels which were mostly filled with thrombi of varying age.

Liver: The acinous liver structure was preserved. Here and there the liver parenchyma showed small necroses with accumulations of up to about 20 leukocytes. The number of leukocytes within the liver capillaries seemed to be generally increased as well. Within the liver, too, marked focal vascular changes were observed which were localized primarily to the arteries. All the layers of these arteries were necrotic and the lumen was filled with a thrombus which contained abundant leukocytes, infiltrating also the necrotic vascular walls and the surrounding tissue. The inflammation had in some places invaded the contiguous branch of the vena porta, causing the formation of a leukocyte-rich thrombus in that part of the circumference of the vein facing the artery (Fig. 9).

The heart, pancreas and gastro-enteric tract showed normal conditions.

The case is a 57-year-old woman with a mild acute otitis, which did not, however, explain the affected general condition and high fever. In chest radiograms several rounded densities up to the size of a mandarine were discovered and interpreted as tumor metastases (Fig. 1). Urography showed an expansive process in the left kidney; clinically the case was diagnosed as a nephroma with pulmonary metastases. The autopsy, however, showed the expansive process within the left kidney to be a serous cyst. The densities in the lungs ascertained by X-ray corresponded to greyish yellow, rounded, necrotic foci, which were more or less sharply defined (Fig. 2). The autopsy revealed also multiple anemic infarcts in the kidneys (Fig. 3) together with thrombi in the connected renal vessels, a septic swelling of the spleen with multiple infarcts, and a general toxic condition of the organs. No tumor could be observed anywhere.

The microscopical examination showed that the foci within the lungs consisted of a necrotic tissue. Adjacent vessels, both arteries and

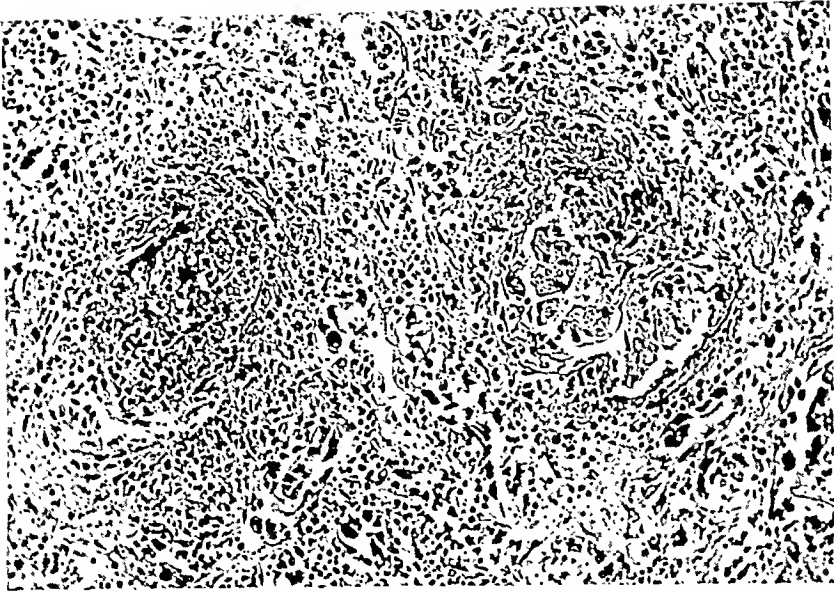


Fig. 8.

To the right fibrinoid necrosis in a few glomerular loops; to the left degenerated glomerulus with periglomerular granuloma.

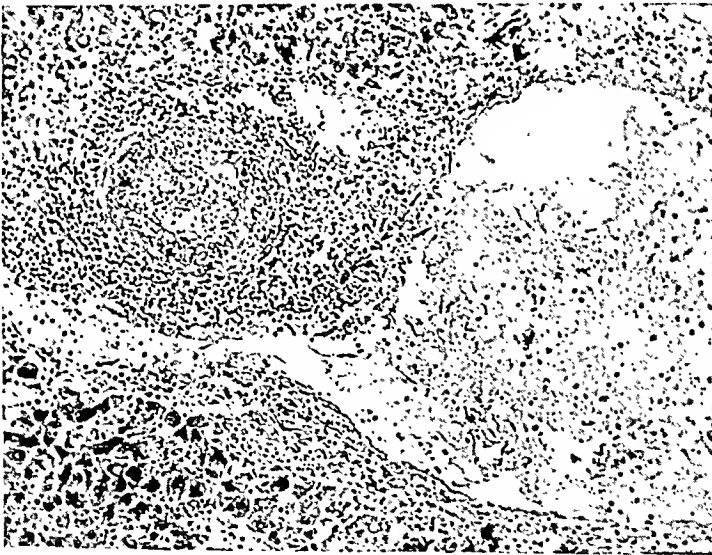


Fig. 9.

Necrotic arteritis in the hepatic artery, spreading to the portal vein; secondary wall thrombus in the vein.

veins, showed marked changes in the form of thickening of the intima caused by an edematous swelling of the subendothelial tissue and a sparse infiltration of inflammatory cells (Figs 4, 5, and 6). Some vessels showed a total sometimes fibrinoid necrosis of the walls and a diffuse infiltration of neutrophile leukocytes. Many of the changed vessels were filled with thrombi of various age and rich in leukocytes. It was characteristic that these alterations in the pulmonary vessels were found only near the pulmonary foci and sometimes only on that part of the vessel facing the focus. Similar inflammatory changes were found in several renal arteries (Fig. 7). The arteries corresponding to the large anemic infarcts were thrombosed and the veins were the site of secondary thrombi. Several glomeruli showed partial or total necrosis, and were transformed to small granulomas (Fig. 8). The spleen showed vascular changes and thrombi. Inflammatory focal changes of the arteries were found also in the liver in combination with thrombus formation (Fig. 9).

Discussion.

The clinical diagnosis of this case was made on the basis of the roentgen examination. The chest radiogram showed several rounded densities, often sharply defined. Urography showed a fist-sized expansive process in the upper left kidney and the diagnosis was nephromata with pulmonary metastases. The post-mortem examination, however, showed the expansive process to consist of a solitary renal cyst containing a clear thin liquid. This case illustrates the great difficulty in forming a differential diagnosis of renal tumors and solitary renal cysts. The author has demonstrated in a report on the roentgen diagnosis of the renal tumors (*Johansson, 1946*) that it is impossible to distinguish between a nephromata and a solitary cyst by means of roentgen, unless an invasion of the renal pelvis or the presence of metastases can be proved.

Clinically, this case agreed well with the previously described cases of Wegener's granulomatosis. The patient is taken ill with symptoms of otitis after which pneumonic symptoms set in. The morbid picture, appeared as a septic condition with a fatal outcome after about a month. One of *Postel & Laas'* cases (fatal after 4 weeks) was taken ill with otitis. The mild ear symptoms, however, did not explain the patient's septic condition. A chest radiogram was taken and showed cloudy densities. Their case is thus very similar to the one reported here. The only non-protein nitrogen determination made — 12 days before death — showed a normal value. It is probable that the non-protein nitrogen was raised later on, in view of the pronounced bilateral renal changes. It is also possible that no uremic state developed because the patient died before the periglomerular granulomas had time to become so numerous as to cause renal insufficiency.

The case agrees well also pathologically-anatomically with the

earlier described cases. Features in common were necrotic pneumonic changes and pronounced generalized inflammatory vascular changes of exsudative, necrotic, or proliferative type. In the kidneys there were moreover periglomerular granulomas with the typical aspect of Wegener's granulomatosis.

The anatomical findings in this case were dominated partly by the multiple necrotic lung foci, partly by the vascular changes especially in the lungs. The first problem to be solved is their mutual relationship. Are the necroses in the lung parenchyma a result of the vascular changes or have these been instead produced by primary necrotic processes in the parenchyma? It is obvious that there is a close causal connection between them; the vascular changes and necrotic foci do not occur isolated but always together. Both *Wegener* and *Postel & Laas* observed that the inflammatory vascular changes were most obvious within and close to the necrotic foci in the lungs. In *Wegener's* case there were inflammatory changes also in vessels which were not in the immediate vicinity of the pulmonary foci. In *Postel & Laas'* two cases, on the other hand, as in the case reported here, no vascular changes occurred outside the necrotic foci. *Ringertz, Lindsay et al.* have given no detailed description of the relation between the vascular and lung changes. Both *Wegener* and *Postel & Laas* consider the vascular changes to be primary and the necroses in the lungs to be secondary infarcts which have been subsequently infected. As pointed out by *Damble* (1930) and *Volland* (1935) there are in periarteritis nodosa in the lungs usually no other changes than those connected with the vascular system.

In order to determine the relationship between the vascular changes and the necroses in the lungs, special attention should be given to the following observation: *the affected vessels are strikingly often arranged in a ring in the periphery of the necrotic foci.* This circumstance speaks in favor of the view that the vascular changes, which occur in both arteries and veins, in this case have been provoked by an agent working centrifugally from the necrotic lung processes. This assumption is also consistent with the form of the foci. If the vascular processes had been primary, at least part of the foci ought to have the triangular shape characteristic of infarcts, considering the vascular ramification in the lungs. This was not the case, however. Moreover, some vessels showed inflammatory changes only on the part of the circumference which was adjacent to the necrotic focus. The vascular changes may, however, have contributed to the extension of the necrotic lung processes.

The outstanding finding in this case thus seems to be inflammatory changes in the pulmonary parenchyma, similar to necrotic pneumonic foci. The fibrinous exudate in the alveoli and the infiltration of inflammatory cells, especially neutrophile leukocytes, within the foci and especially in the adjacent pulmonary parenchyma, speak in favor

of this theory. The form of the foci is also similar to that in bronchopneumonias.

The inflammatory changes in the pulmonary vessels do not exclude the assumption that the necrotic foci are of pneumonic nature. Thus inflammatory changes in the pulmonary vessels occur both in lobular and bronchopneumonias and are probably more common than was previously assumed (*Borst* 1918, *Fossel* 1941).

The generalized vascular changes which occur in the vessels of the spleen and in the arteries of liver and kidneys may be regarded as an expression of a violent allergic reaction, produced by the infection in the lungs. The fibrinoid changes and necroses in the vascular walls, as well as the periglomerular granulomas, agree in their general type with the changes found in hypersensitive conditions.

The starting point of the pathological processes in the case reported here has thus been the multiple necrotic bronchopneumonias which have produced local vascular changes. The severe changes in the vessels of both spleen, liver, and kidneys, may have arisen as a hypersensitivity reaction against the infection in the lungs. The fact that the morbid picture was considerably shorter in this case than in *Wegener's* is probably due to the fact that the primary infection was localized to the lungs and not to the upper respiratory tracts.

Summary.

A report is given of a 57-year-old woman, who died after a rather short febrile disease. The roentgenological examination revealed multiple rounded sharply defined foci in the lungs as well as an expansive process in one kidney. The clinical diagnosis was nephroma with lung metastases. The autopsy showed a serous renal cyst and multiple necrotizing pneumonias. Focal inflammatory vascular changes were observed in the lungs, liver, spleen, and kidneys, localized especially to the intima and sometimes combined with thrombi. In the kidneys, moreover, infarcts and periglomerular granulomas were found. The case is linked with a pathological condition reported earlier by *Wegener* (*Wegener's* granulomatosis).

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A REPORT ON CONGENITAL TOXOPLASMOSIS

By Olof Smitt and Sten Winblad.

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Toxoplasmosis is caused by a protozoan parasite, *Toxoplasma*, which is believed to be similar to *Leishmania*, but apparently it has not been classified definitely as yet. Toxoplasmosis has been recognized as a disease in animals since 1908 when *Nicolle & Manceaux* and *Splendore* reported two cases. In 1939 *Wolf* and his co-workers were able to establish that the genus *Toxoplasma* is pathogenic also for humans. Previously several cases had been described which might have been toxoplasmosis and in some of these similar parasites were demonstrated. *Castellani* (1914) and *Fedorovitch* (1916) reported two identical cases of a disease in children, characterized by prolonged fever, anemia, and splenomegaly, where the investigators found parasites, believed to be *Toxoplasma*, in the blood. *Chalmers* and *Kamar* (1920) also reported similar parasites in a splenic film from an adult who suffered from prolonged fever. *Jankú* described in 1923 a case of simple microphthalmus in an infant with increasing hydrocephalus and convulsions. He observed parasitic cysts in chorioretinitic foci and believed the parasites to belong to Sporozoa. *Levaditi*, however, suggested that they were *Toxoplasma*. Similar parasites are described by *Torres* (1927).

In 1939, *Wolf* demonstrated a case of congenital encephalo-myelitis in a newborn, caused by *Toxoplasma*, and could confirm this diagnosis by experimental passage of the human infection to animals. In the same year *Sabin* succeeded in establishing that *Toxoplasma* found in humans were neither biologically nor immunologically different from *Toxoplasma* isolated from guinea pigs. Since these observations, our knowledge of toxoplasmosis has increased. Various clinical manifestations of this infection have been observed especially by *Wolf* and his

coworkers. Numerous cases reported as uncertain have now been classified as more or less certain cases of this disease (*Fishll*, 1897 and 1899; *Wohlwill*, 1921; *Coulon*, 1929; *de Lange*, 1929; *Brandt*, 1934; *Hertig*, 1934; *Richter*, 1936).

Toxoplasmosis seems to occur within several different animal species as dogs, cats, sheep, guinea pigs, voles, squirrels, rats, minks, foxes, and pidgcons. The infection seems to be widespread throughout the world and cases have now been reported from all continents. The source of the infection in man is with all probability to be found among the animals. Occasionally the infected animals become sick but sometimes the infection follows a subclinical course resulting in healthy toxoplasma carriers. The clinical symptoms in animals are atypical, but loss of weight, bloody diarrhea, and symptoms from the respiratory tract often occur.

Experimental infection can be produced by: intracutaneous, subcutaneous, intravenous, intraperitoneal, intracerebral, or intranasal routes. It is not clear whether biting insects can transmit the disease but midges, lice, or ticks are considered by *Sabin* as possible carriers of toxoplasmic infection. The urine and feces of infected animals, especially dogs and cats, have also been found to contain *Toxoplasma*. Our present knowledge of the clinical and pathological manifestations of toxoplasmosis in man permits a differentiation of 2 types: one congenital and one acquired.

In *congenital toxoplasmosis* the central nervous system shows the chief signs of infection. According to *Sabin* there is a tetrad of clinical signs: internal hydrocephalus or microcephaly, chorioretinitis, convulsions, and roentgenological evidence of cerebral calcification. The destruction in the brain is often extensive, consisting of miliary granulomas or larger inflammatory areas with necrosis. The inflammatory processes seem to be most intense in the cortex, basal ganglia, or periventricular tissue. Meninges and ependyma show often areas of inflammation which may be the cause of the internal hydrocephalus. An early hydrocephalus originating *in utero* may lead to difficulties in delivery (*Wolf & al.*). In a prolonged inflammation a calcification of the necrotic areas often occurs and large widespread calcified zones may arise within the brain. *Toxoplasma* organisms are to be found in the inflammatory areas where they occur in inflammatory cells, in the vascular endothelium for which they seem to have a great affinity, and occasionally also in nerve cells. No *Toxoplasma* are found in the necrotic areas and it has been pointed out by *Wolf*, *Cowen & Paige* and *Zuelzer* that *Toxoplasma* are difficult to find in older processes as they are easily disintegrated and calcified. A chorioretinitis may occur, generally in both eyes, mainly localized to the fovea centralis. The granulation may invade the vitreous body causing microphthalmia of one or both eyes.

The congenital disease may even attack other organs. Lesions have

been found in the lungs in the form of interstitial pneumonia with *Toxoplasma* in the alveolar epithelium; in the heart with granulomas in the myocardium; in the adrenals, thyroid gland, ovaries, testis, kidneys, as well as in both striated and smooth muscles (*Paige, Cowen & Wolf; Zuelzer; Pratt-Thomas & Cannon* and others). According to *Zuelzer* hepatosplenomegaly is often found in infants with toxoplasmosis and sometimes an extramedullary hematopoiesis.

In smears and thin sections *Toxoplasma* appear induced in the vascular endothelial cells and in nerve and muscular cells. They are apparently mainly intracellular protozoan parasites, and are often observed in clusters or »cysts«, where they may completely fill or deform the host cells. In tissue sections the characteristic shape with nuclear chromatin and thin surrounding plasma is not always seen.

Toxoplasmic infection seems to stimulate the appearance of antibodies, which can be demonstrated in serum. The antibodies have a neutralizing effect when injected intracutaneously in rabbits in combination with a *Toxoplasma* strain of high virulence. They are, however, very labile, diminishing in serum which is not preserved in the frozen state. The serological test should therefore be carried out as soon as possible after the sample has been taken. In congenital toxoplasmosis the blood of the mother as well as that of the child has been found to contain antibodies. Often no antibodies can be found although the disease has been definitely ascertained. *Sabin* has shown that infected apes may lose their antibodies even 6 weeks after the onset of infection. *Heidelman* has demonstrated that serum from normal individuals who, as far as is known, have not been infected with *Toxoplasma*, sometimes may give a positive reaction. A positive serological test alone is thus not a certain indication that an infection has occurred. Negative reactions, on the other hand, can be obtained in well defined infections.

The chief clinical signs in congenital toxoplasmosis are usually the destructive lesions in the brain. The development of the skull is normal only in mild cases. A rapid hydrocephalus or sometimes microcephaly, is present in typical cases and the child has clear neurologic symptoms — convulsions or tonic disturbances, from the onset. The outcome is generally fatal after a short time, often with a complicating secondary infection. If the damage is only slight at birth, the symptoms may not manifest themselves until late in infancy or in childhood. The clinical manifestations are then convulsions, spastic conditions, and mental defects. Chorioretinitis occurs at the same time, causing deficient sight and nystagmus. Sometimes a defectively healed chorioretinitis is found, consisting of yellowish white, atrophied areas in the retina, limited by dark pigment. Microphthalmia, enophthalmus, and atrophy of the optic nerve or membranous formations in the vitreous body are often present (*Koch & Wolf; Cowen & Paige; Heath & Zuelzer*). Roentgenographical evidence of calcifications in the brain and positive serological

findings are also important for the diagnosis. In the cerebrospinal fluid of acute cases xanthochromia and elevated albumin values are found, and occasionally pleocytosis. Toxoplasma have even been observed in the fluid. Other symptoms to be mentioned are jaundice and enlargement of liver and spleen. The disease has occasionally been diagnosed as erythroblastosis foetalis (*Steiner & Kaump*).

The acquired infection of toxoplasmosis occurs in both children and adults. It takes the form of an acute encephalitis or acute febrile illness with exanthema. It begins with a sudden rise in temperature and chills. A maculopapular rash covering the whole body, except the scalp, the palms of the hands, and the soles of the feet is a fairly early symptom, often in connection with signs of pneumonia. The mortality rate is relatively high (*Syvertsen & Slavin*).

A subclinical form of the disease seems to be fairly harmless. Mothers transmitting the infection to the foetus do not as a rule present any serious symptoms although showing positive serological reactions. The subclinical form must be as common as the congenital form of the disease.

The distribution of Toxoplasma in animals is widespread and world-wide. About 40 human cases have been reported so far and new cases are constantly being published in the literature. In Europe instances of the disease have been reported in Czecho-Slovakia, France, the Netherlands, Switzerland, Italy, and England. Until 1946 the disease had not been observed in Scandinavia. During that year the present authors had difficulty in diagnosing the disease of an infant with some symptoms of congenital toxoplasmosis. At first it did not seem very likely that this case was a disease which had not been previously observed in this country. Shortly afterwards, however, we learnt from *Magnusson & Wahlgren* that they had observed a similar case in Stockholm and also demonstrated toxoplasmosis in older microscopic preparations. Later, at the Scandinavian Meeting of Pathology in 1947, *Sjölte* described a case of toxoplasmosis in a dog in Copenhagen; the disease has also been reported recently from Gothenburg (*Mellgren*). In view of the fact that this disease appears to occur in Scandinavia as well, the following case seems worthy of mention.

Report of case.

Journ. No. 782/46. The parents and their 2 other children are healthy. Wassermann's test of the parents was negative. Their Rh blood-type gives no reason to suspect erythroblastosis. There was no special contact with animals. In the third month of pregnancy the mother had a »severe cold« with nasal discharge and cough. During the last months of pregnancy she suffered from nasal obstruction but was otherwise in good health.

The infant, a girl weighing 2,460 gm., was born on October 16, 1946, 3 weeks before the proper time. The cause of the premature delivery was unknown. The delivery was normal. The amount of amniotic fluid was, however, unusually large — 4 litres. The placenta was large, weighing 880 gm. Immediately after birth the liver and spleen of the infant were found to be

enlarged. The day after birth a slight jaundice appeared and several small bleedings in the skin were noticed. The infant was admitted to Flensburg Children's Hospital when 6 days old. She weighed on admittance 2,100 gm. having lost 360 gm. The length was 45.5 cm. and the circumference of the head was 32 cm. The child seemed sickly on account of its greyish cyanotic, dirty color. The jaundice was moderate. No serious bleedings or exanthema were seen. The anterior fontanel was slightly depressed and the bones of the skull were intercalated. The reflexes were normal, even Moro reflex. Eye slits and eye-balls were strikingly small and it was definitely a case of microphthalmus. The thoracic organs seemed quite normal. The liver was considerably enlarged, one fingersbreadth below the costal margin. Also the spleen was enlarged, extending down to the umbilical region (Fig. 1).



Fig. 1.

The infant with the much enlarged liver and spleen.

The increase in weight during the stay at the hospital was slight, only 140 gm. the first month. The patient had to be fed by means of a stomach tube and vomited often. The general condition became gradually worse. The temperature was normal at first, but the erythrocyte sedimentation rate was 6 mm./hour (microreaction according to Landau). The blood values were initially high, red blood cells; 4,160,000, Hemoglobin; 115 %, decreasing soon to 2,450,000 and 64 % respectively. White blood cells were at first 7,600 and dropped to 3,900. The differential count showed no remarkable changes. The anemia increased and in spite of the administration of two intraperitoneal blood-transfusions of 35 and 45 ml., the blood values remained low and at death there were 1,400,000 red blood cells, 50 % hemoglobin, and 4,900 white blood cells. The sedimentation rate rose slowly to 38 mm. The jaundice disappeared after one month and the size of the liver seemed to diminish during the course of the illness. The child received mother's milk during the entire period. Wassermann's test was negative and blood culture showed no growth of bacteria. A few weeks after birth a few prominences developed on the back of the head, gradually growing to the size of a hazelnut and fluctuating. When punctured they yielded pus from which *Staphylococcus aureus* was obtained in pure culture. Penicillin, 5000 units 8 times per day, was administered and a slight but transient improvement was noticed.

The baby gradually became worse, and died in the eighth week of life. The temperature regulation was poor in spite of satisfactory incubator treat-

ment and deviations between subnormal and slightly febrile temperature were noted. In the last week before death it reached a maximum of 40.5° C. Blue cyanotic spots appeared on face and abdomen during the last two weeks.

The case can be summarized as follows: a case of serious infection and congenital enlargement of liver and spleen. The erythrocyte sedimentation rate was considerably increased from the beginning, but the blood picture was not typical of an infection. Infection by Staphylococci was established but the therapeutic effect of penicillin was only slight. Erythroblastosis and lues could be eliminated. The problem was whether the infection was congenital and might be the cause of the enlargement of liver and spleen. The only bacterial finding was Staphylococcus aureus, which is obviously pathogenic but can hardly be considered the cause of an infection *in utero*.

Report of necropsy.

The case was that of a tiny girl, evidently emaciated and weighing 2400 gm.

At the back of the neck two flabby edematous non-suppurative swellings were present. When the bones of the skull were taken away ample cloudy liquid was discharged. The brain was soft and upon dissection showed grave lesions. Hydrocephalus was present, especially in the lateral ventricles of the brain. The cortex of the frontal part of the brain showed microgyria. A yellow necrotic fibrinous tissue surrounded all the ventricles except the fourth. These yellow necrotic areas occurred also as foci within the substance of the hemispheres in which small central cystical formations were found. Ample calcification appeared within the necrotic zones. The outlines of the ventricular system were obscured and the topography damaged by the necroses. The cerebellum and oblongata appeared quite normal. The meninges were only slightly thickened and no signs of actual meningitis were observed.

The heart was normal, without malformations. The myocardium appeared normal and there were no signs of endocarditis.

The mucous membrane of the trachea and bronchii was slightly reddened and coated with a purulent secretion. The upper pulmonary lobes contained air and were apparently normal while the lower lobes were cyanotic and inflamed. The small bronchial lumina yielded on pressure a cloudy liquid. The picture was that of a bronchopneumonia of aspiration type.

The picture of the abdomen was dominated by the greatly enlarged liver and, at the left and below the liver, by a very much enlarged spleen. The latter weighed 82 gm. and upon dissection was light red, homogenous, and somewhat soft but not degenerated or softened as in a sepsis. The liver weighed 195 gm. and was somewhat greenish and knotty as in cirrhosis. A section showed small pseudo-lobes and partly fibrous areas. The pancreas seemed to be normal as well as the biliary ducts.

The intestines with mesenterium, adrenals, and kidneys were normal. The genitals were also of normal appearance.

Microscopic findings.

Brain: The yellowish white necrotic areas were seen under the microscope to be zones of granular tissue with numerous cells, calcifications, and necrotic cerebral tissue. The calcifications consisted of aggregations of gritty calcified material, crowded together and forming large clumps and bands penetrating the tissue (Fig. 2). Bordering these zones inflammation occurred with all kinds of inflammatory cells. Abundant plasma cells and sometimes also eosinophile leukocytes were found, however. Within a zone outside the calcified clumps were small foci of calcified grains, each of them only 1/10 the size of the cell nuclei. These grains were the same size as that of



Fig. 2.

Calcifications in the necrotic cerebral tissue near the ventricular wall.

Toxoplasma described, but they were evidently calcified and had no definite morphological resemblance to *Toxoplasma* (Fig. 3). At the same time there was a few bacterias of the coccus type. These findings were sparse, however, and lacking in most areas examined. In another zone outside the calcifications, small oval particles were present, most often in groups within

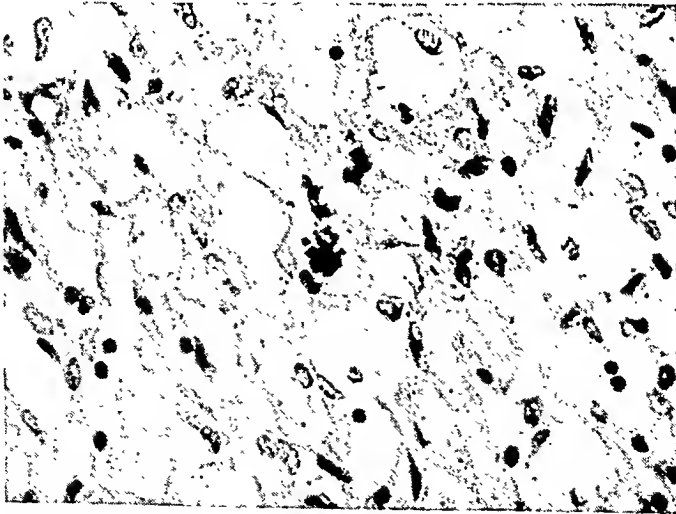


Fig. 3.

Calcified focus in the cerebral tissue outside the large calcified areas.

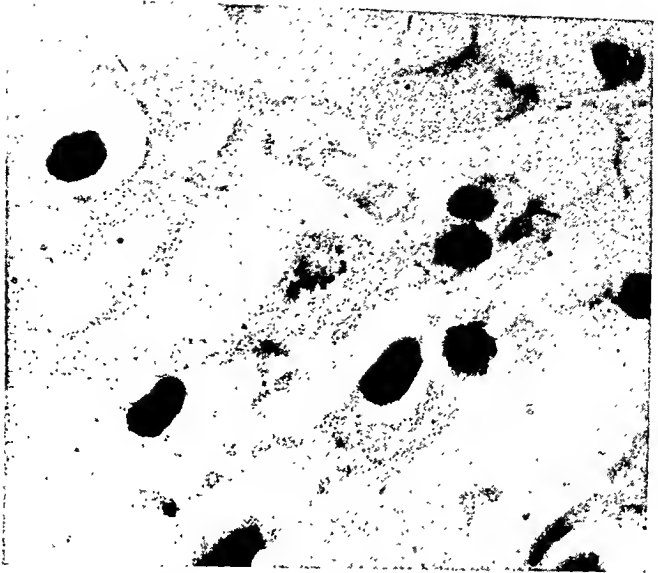


Fig. 4.

Groups of particles resembling *Toxoplasma* in the inflamed and partly necrotic brain tissue outside the calcified zone ($\times 2000$).

the cell plasma, either of the endothelial cells or of other cells difficult to identify. They were ovoid and occasionally a central nucleus could be discerned. They were similar to *Toxoplasma* and were interpreted as such. No calcification was observed within these particles, presented in Figs 4—6. They were easily distinguished from the numerous degenerating cellular nuclei found within this granular tissue. It was interesting to note that they were situated in a zone outside of the central granular tissue with its necrosis and calcification.

Liver: There was widespread inflammation with gross destruction of

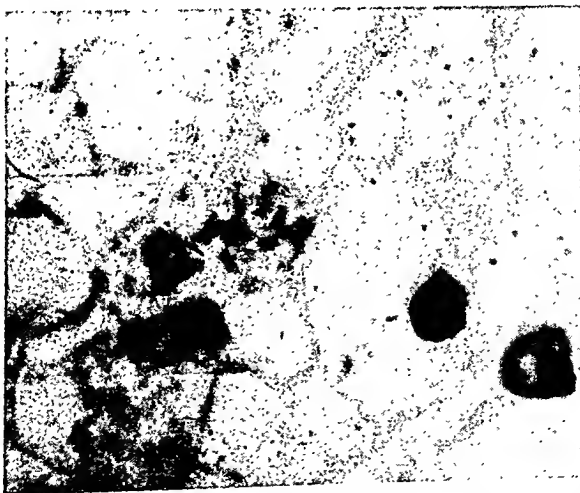


Fig. 5.

Groups of particles resembling *Toxoplasma* in the cerebral tissue ($\times 2000$).

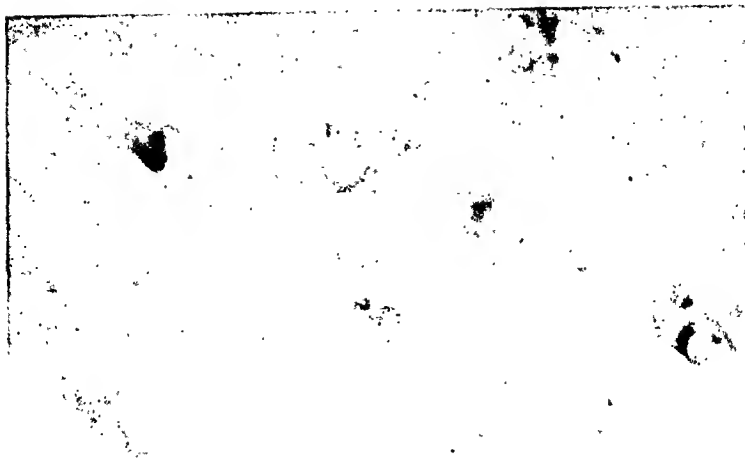


Fig. 6.

Groups of particles resembling *Toxoplasma* in the cerebral tissue ($\times 2000$).

the hepatic tissue and cirrhotic transformation of the parenchyma (Fig. 7). The inflammation was most intense in the periportal zones where there was a variegated granular tissue with numerous eosinophile leukocytes. The inflammatory picture does not resemble any other specific inflammation. Within some tissue cells an inflammation of small particles was found, ovoid-shaped and similar to *Toxoplasma* (Fig. 8). They were very sparse, however, and only a few cells with inclusions of this kind were found.

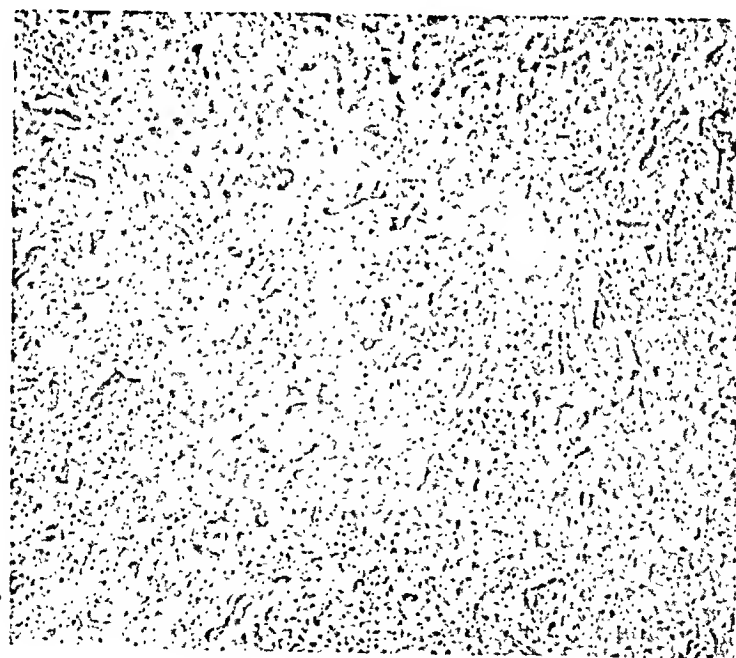


Fig. 7.

Hepatic tissue with gross inflammation and degeneration of the parenchyma.

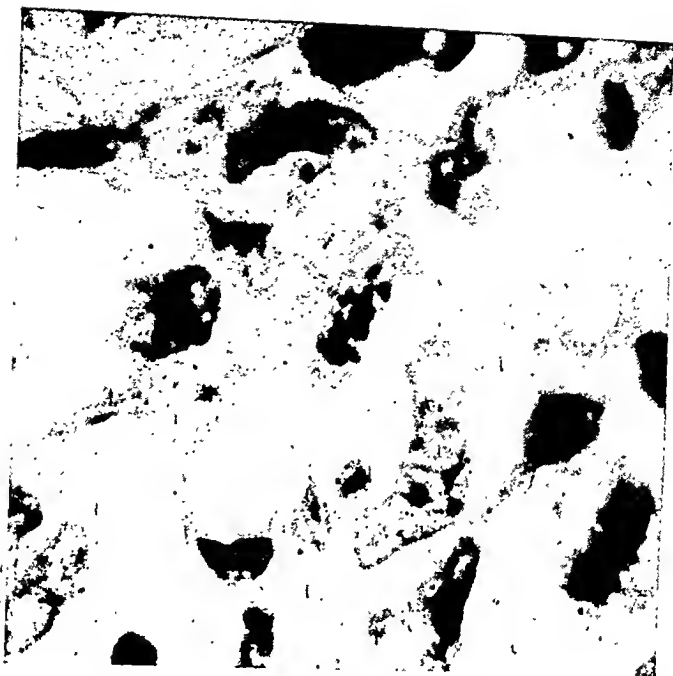


Fig. 8.

Inclusions resembling *Toxoplasma* within the inflammatory granulation tissue of the liver ($\times 2000$).

Spleen: The spleen showed signs of circulatory stasis with filled pulp chambers. No actual inflammation was present and no *Toxoplasma* particles were found.

Within the myocardium, renal tissue, bone marrow and adrenals there was no evidence of *Toxoplasma* particles. There was no sign of myocarditis of the myocardium. Eosinophile leukocytes were abundant in the bone marrow.

Serological examination.

A sample of the mother's blood was sent to Doc. S. Gard at Statens Bakteriologiska Laboratorium to be examined for the presence of antibodies by testing the neutralizing effect of the serum against a *Toxoplasma* strain. In intracutaneous experiments on rabbits the serum neutralized 5 but not 25 reactive doses. Intracerebral experiments on mice gave a similar result, indicating that the mother's blood contained a slight demonstrable amount of neutralizing antibodies. The serum used in these experiments was not completely fresh but had been stored for several days at -16° C. It is quite probable that the serum originally contained a greater amount of antibodies, for, as has been pointed out above, antibodies in serum are very labile. The serological examination did not take place until 5 months after the birth of the child and it is probable, too, that the antibodies decreased during this time.

The case in question is one of congenital infection with congenital enlargement of the liver and spleen, an early increased sedimentation rate, obvious signs of serious infection, and general debility. Further examination has shown microphthalmia, severe brain lesions with

widespread necroses and calcifications, cirrhosis of the liver, microscopic inclusion particles resembling *Toxoplasma* in the cerebral tissue and in the liver, as well as a faintly positive serological reaction for Toxoplasmosis in the mother's serum.

Discussion.

The widespread cerebral calcifications dominated the inflammatory picture in the case reported above. They are the most characteristic feature in toxoplasmosis but may occur also in other diseases: cerebral tumors, such as craniopharyngeomas and anginomas venosum, Sturge-Weber syndromes, tuberous sclerosis, »epilepsy with cerebral calcifications«, subdural hematomas, tuberculomas, cysticercosis, and intracerebral hematomas may occasionally show cerebral calcifications. Among these tuberous sclerosis may possibly be discussed as a differential diagnosis in the case reported. This disease appears, however, most often in older children, and is not quite similar to the one described above, although the calcifications in tuberous sclerosis are also mainly localized to the peripheral areas of the ventricular system.

The definite eosinophile of the tissues was remarkable and occurred both in the encephalitic and hepatic inflammation. *Pratt-Thomas* and *Cannon* have in a case of toxoplasmosis found fairly numerous eosinophile myelocytes in the liver. In our case no higher degree of eosinophilia was observed in the blood (6—8 %), but it was fairly high in the bone marrow. The eosinophilia in these cases may possibly have the same causes as those observed in cases of such parasites as intestinal worms.

According to the literature inflammation in the liver is a rather common feature of congenital toxoplasmosis, usually occurring as focal inflammatory lesions, small necroses, and biliary stasis (*Pratt-Thomas & Cannon*; *Zuelzer*). Jaundice and residual hematopoiesis are often found, too. The liver in our case showed a grave inflammation with an early cirrhotic transformation of the organ and *Toxoplasma*-resembling inclusions in some cells.

The eye was unfortunately not preserved for histological examination. The manifested microphthalmia speaks, however, in favour of the diagnosis toxoplasmosis.

With regard to therapy *Sabin* mentions that almost all antiprotozoal drugs have been tried without effect. Sulfathiazole and sulfapyridine, have no therapeutic effect *in vitro* against *Toxoplasma* but were found to exert an inhibiting and sometimes curative effect on the infection. *Robinson* (1947) has recently described a case of toxoplasmosis in a 9-year old girl with clear symptoms of encephalo-meningitis and positive findings of *Toxoplasma* in the liquor. After treatment with sulfathiazole for 18 days, divided into two periods, there was a total regression of the infection. It seems thus worth while to try sulfathiazole and emetine as a therapeutic agent. The prospect of improve-

ment must then be greater in acquired toxoplasmosis than in the congenital form where extensive damages have already been made on the central nervous system.

It is interesting to note that this disease seems to exist also in Scandinavia. New cases of toxoplasmosis may be discovered if attention is paid to symptoms of *Toxoplasma* type and serological tests are carried out in suspected cases. It is hardly likely that the disease is new in our country — it has probably existed but not been diagnosed. The findings made by *Magnusson & Wahlgren* on preparations from children who have died with congenital cerebral calcifications bear witness to this.

Summary.

A case believed to be congenital toxoplasmosis is reported. A newborn girl had an early increased sedimentation rate, hepatosplenomegaly, and microphthalmia, combined with general debility. Signs of grave infection were observed during the two months of life with irregular temperature and increased sedimentation rate, but no reaction in the blood in the form of leukocytosis. A secondary *Staphylococcus aureus* infection was also present.

On autopsy gross damages were observed in the brain with necrosis and calcifications, together with grave inflammation of the liver. Inflammatory conditions resembling those in previously described cases of congenital toxoplasmosis were found in microscopical preparations. In the cerebral tissue several small particles were present resembling *Toxoplasma*. Serum taken from the mother showed antibodies against *Toxoplasma*.

A survey is given over the clinic and pathology of toxoplasmosis, especially the congenital form.

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THE INFLUENCE OF WOOL FAT ON THE HEALING OF WOUNDS

By Gösta Lindquist.

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I. Introduction.

Irrespective of the methods that are otherwise used in the treatment of open skin wounds, the use of a protective dressing seems to be inevitable. The natural wound healing, i. e. the healing under the formation of a scab without the presence of a dressing, thus practically never occurs in the normal wound therapy. In investigations on the manner and the time of the healing of a wound, »wound healing with dry dressing« ought therefore to be regarded as the normal way.

In an experimental investigation on the wound healing in the white rat (1946) I have made, *inter alia*, comparative experiments on the healing of open wounds from a macroscopic and a microscopical point of view. The wounds were treated with dry dressing, vaseline, wool fat, and wool fat containing 25 % of water (= lanolin) and 50 % of water.

The macroscopic examination yielded the result that vaseline accelerated while wool fat (with or without an addition of water) retarded the wound healing as compared with dry dressing.

The histological examination of the wounds showed in the main that the granulation tissue in the wounds treated with dry dressing and in those treated with vaseline had a similar look. The collagenous fibres are arranged in certain definite main directions, and the fibrosis proceeds gradually from below towards the surface. The difference is chiefly quantitative; the granulation tissue in the wounds treated with vaseline is more rapidly formed and assumes a considerable thickness. In the wounds treated with wool fat and wool fat containing 25 or 50 % of water a peculiar granulation tissue is formed almost throughout. The collagenous fibres lie here at random, and at an early

stage thick connective-tissue fibres appear close to the surface of the wound. In the intermediate stages (12th—15th day) there appear moreover almost throughout plenty of cavities of different size. When stained in the usual way with sudan in order to establish the presence of fat, they proved to be filled with fat (Fig. 1).

As these cavities filled with fat appeared only in the wounds treated with wool fat, it seemed reasonable to suppose that this fat came from the wool fat on the surface of the wound. But it is a well-known fact that the organism, when subjected to all kinds of toxic influences, reacts with degenerative changes, *inter alia*, with accumulations of fat in the tissues. It might therefore be possible that the fat in these cavities comes from the body itself and that the accumulation has taken place as a reaction of the granulation tissue against the wool fat on the surface of the wound. As the fat of the body and the wool fat will quite naturally be stained in the same way, when the common methods of staining are used, no definite knowledge of this could be attained from the experiments. Other methods must be used. The simplest way of solving the problem ought to be by treating the wounds with stained wool fat and leaving the histological sections unstained. The cavities in the granulation tissue will then be filled either with stained or unstained fat, depending on whether the fat is of an exogenous or endogenous origin. From this starting-point the following experiments have been made.

II. Method and material.

The method used was in the main identical with the one employed in earlier experiments on the healing of wounds with regard to the contraction process. The material consisted of 10 white rats.

All operations and dressings were made under ether narcosis and sterile precautions. On the cranial part of the animal's trunk with the exception of a narrow part on the ventral side, the skin was freed from hair by cutting and subsequent shaving. Immediately after the shaving the epilated skin area was dried with a clean towel, after which two wounds were produced on each animal; they were placed some distance caudally to the scapulae on either side of the spinal column, and as symmetrically as possible. In order to mark the location of the wounds, a circular stamp ca. 18 mm in diameter was used. With a pair of curved scissors, the marked skin with the subjacent part of the *erector spinae* muscle was cut away, so that the bottom of the wound consisted of the loose subcutaneous connective tissue.

The wounds were covered with sterile compresses, on which wool fat was smeared. The wool fat had been stained with a solution of sudan (50 grammes of wool fat + 30 grammes of solution of sudan). In order to protect the wounds and dressings from injury by the animal, a metallic shield was placed on top of the dressing round the trunk of the animal. The shield consisted of a strip of thin plate about

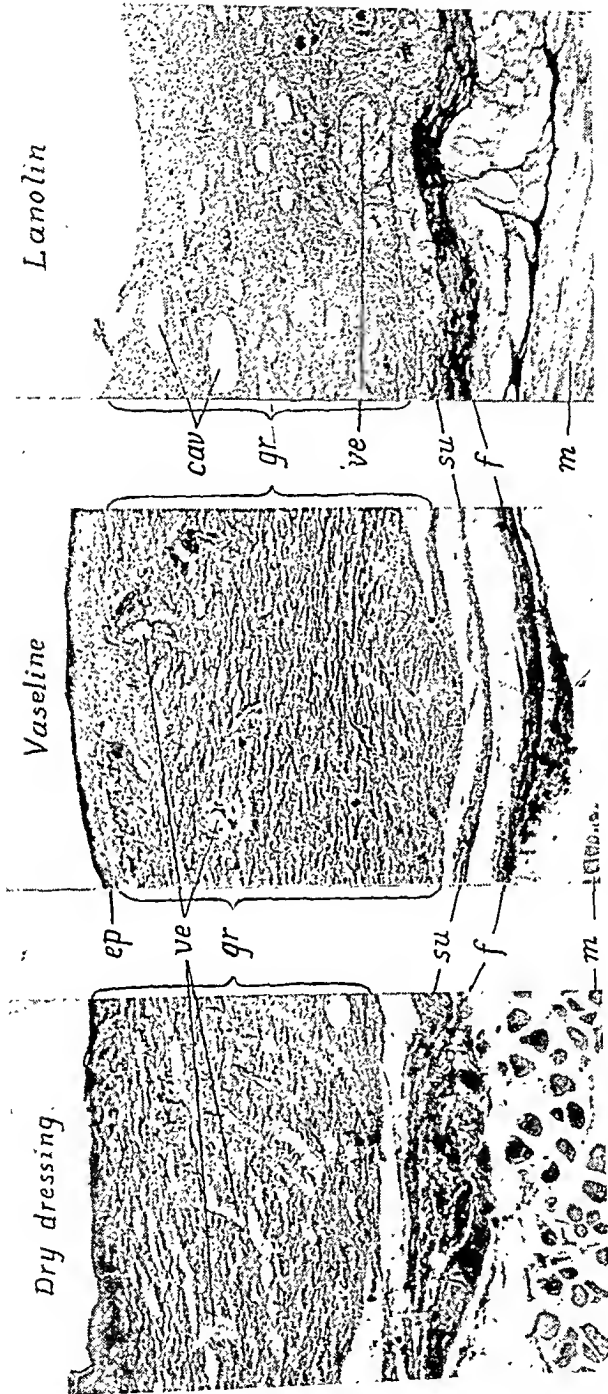


Fig. 1.

Cross sections of wounds treated with dry dressing, vaseline, and lanolin. ep = epithelium. gr = granulation tissue. su = subcutaneous tissue. f = fascia. m = muscles of the trunk. ve = vessels. ca = cavities. / $\times 44$ / Azan.

4.5 cm in breadth and without sharp edges; on the ventrally situated side it was cut out cranially and caudally (Fig. 2). The shield was attached to the animal with mastic on the ventral side. On the dorsal side, where the shield was open, its free ends were held with sticking-plaster.

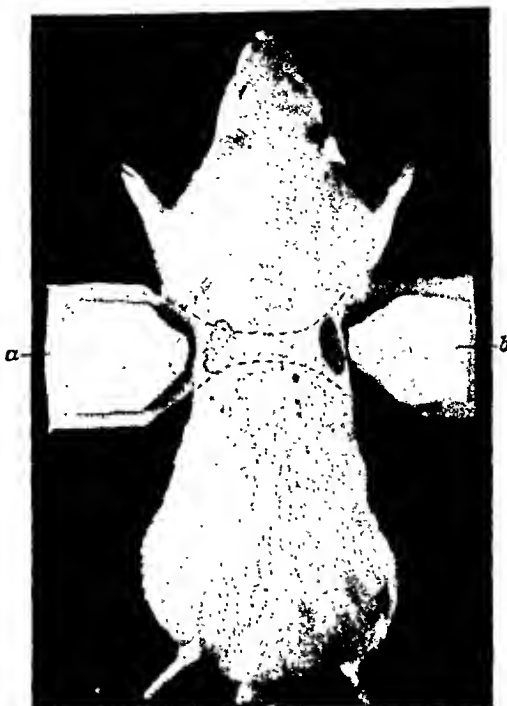


Fig. 2.

Rat with the two wounds on each side of the spinal column and the shifted protective shield with the compresses. The dotted lines mark the part of the shield hidden by the animal. a = free dorsal edge of shield. b = compress.

/ 3 : 4 /

Re-dressing of the wounds was made every third day, when the old compress was replaced by a new ointment compress. On the 9th, the 12th, and the 15th day three animals were killed each time. (One animal died in consequence of an overdose in the narcosis.) The wounds with the immediately surrounding skin were cut out, were mounted on cork sheets and fixed in 10 % formalin. From each wound with the immediately surrounding skin a narrow piece of tissue was cut out, care being taken that the piece corresponded as far as possible to the middle part of the wound. From these pieces sections 50 μ in thickness were cut, as far as possible at right angles to the surface of the wound. From each wound one section was left unstained, while another was stained with hematoxylin and eosin.

III. Results.

On the examination of all the histological sections there appears again the peculiar look of the granulation tissue, that was earlier described, with, *inter alia*, cavities filled with fat. The fat in these cavities is now, however, lightly rosecoloured, and it is easy to distinguish it from the unstained subcutaneously situated fatty cells. The conclusion seems fairly obvious that the fat in these cavities has come from the wool fat of the wound surface in the granulation tissue. It has accumulated here as foreign bodies and as such contributes to retarding the wound healing.

Although no toxic influence on the wounds seems to exist, wool fat ought, according to these experiments, not to be used as an ointment base. As it has too often been pointed out as one of the merits of wool fat as an ointment base, that it absorbs water, I want to emphasize, in this connection, that wool fat absorbs water only through an emulga-tion process, *i. e.* the two substances are vigorously mixed with one another. Resorption of liquid matter from the wound surface to the ointment base because it, *inter alia*, contains wool fat, is therefore practically excluded.

Summary.

If wounds, experimentally produced on the skin of the white rat, are treated with wool-fat (or an ointment base containing wool-fat and water), the wool-fat is absorbed by the granulation tissue and forms extraneous bodies there, that seem to have a retarding influence on the wound healing.

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EXPERIMENTS ON TRANSMISSION OF INFECTIOUS HEPATITIS TO GUINEA-PIGS

By *M. Jersild* and *P. Krag*.

(Received for publication April 3rd. 1948.)

In 1944—45 *Verlinde*, *Boer* and *van Genderen* published their results concerning experimental hepatitis infection on undernourished guinea-pigs*), registering fever (and in some cases death) as signs of established infections. Microscopically liver-lesions consisting of cell degenerations and sometimes hyalin-necrotic foci were found, and serologically both complement-fixating and neutralizing antibodies were demonstrated.

At the State Serum Institute experiments were undertaken from November 1946 to May 1947 to reproduce these extraordinarily interesting results.

Technique.

Normal guinea-pigs weighing from 250 to 300 grammes were kept on their starting-weight on a diet consisting of: 35 ml of a cake baked of wheat-bran 4.5 kilogrammes, oat-grits 2.5 kilogrammes, potato-meal 3.0 kilogrammes, chaffs and water. Besides they got from 5 to 25 grammes of boiled carrots a day to keep their weight constant. As protective food 4 drops of a 5 % solution of ascorbic acid in 1 % citric acid in water, and 3 drops cod liver oil (containing 600 int. units vitamin A) were given. The weight was controlled every two or three days, the temperature measured twice a day.

In comparison guinea-pigs getting normal food would increase their weight from 300 to 400 grammes a month.

*) Insufficient nutrition caused by the shortage of food during 1942—43 in the Netherlands.

The animals were inoculated intraperitoneally once with 0.5 ml of serum or suspension of liver-tissue from patients with hepatitis.

One half of the animals were females. Regarding the fact (Jersild) that most of the fatal Danish cases of hepatitis occurred in women during or after the menopause most of the female guinea-pigs had their ovaries removed 4 weeks before the infection-experiments.

Results.

1) 8 guinea-pigs inoculated with liver-suspension from a case of acute hepatitis (the patient died from a gastric hemorrhage after 4 weeks of jaundice) showed no increase of temperature and no liver-lesions.

2) 4 animals inoculated with icterogenic serum*) showed no increase of temperature and no liver-lesions; in one case there was an increase in the number of Kupffer-cells.

3) 16 animals divided in four equal groups were inoculated with serum or liver-suspension from acute and chronic cases of infectious hepatitis, respectively; the acute being from 10 to 15 days old, the chronic cases 2 months and 2 years old. (Liver tissue was obtained by aspiration biopsy, after the technique described by *P. Iversen* and *K. Roholm*).

8 of these animals showed elevation of temperature for a few up to 15 days during the second to fourth week after the inoculation. Among 15 non-inoculated controls (analogously fed and observed) however one case, which showed increased temperature for five days, was observed. The study of temperatures of all the 31 guinea-pigs in this series has revealed that the temperature in 97 % was higher than 38° Celsius before the animals were put on the special diet outlined above, i. e. while they were fed ordinary food consisting of turnips and hay. The undernourishment caused a decline in temperature, only 29 % of the readings being over 38° Celsius. During the weeks following inoculation the inoculated groups of animals showed increasing temperature, 40 to 50 % of the readings being over 38° Celsius; the controls, on the other hand, had further decreasing temperatures, i. e. 17 to 28 per cent of the readings being over 38° Celsius. This difference is statistically significant, but study of the daily readings showed that many of the daily fluctuations appeared simultaneously among the inoculated and the non-inoculated animals, and that changes in the diet necessary for maintenance of the weight level (i. e. addition of boiled carrots) had several times been followed by rises in temperature.

The weight curves showed that the inoculated animals on the day of infection weighed less than the controls (being chosen so to get the most exhausted animals inoculated). It was therefore often necessary

*) Dr. M. Bjorneboe has made the liver-tissue and the icterogenic serum (034 Mac Callum) available for us.

to add more carrots to these exhausted animals' diet, revising the diet every time the animals were weighed. The additions of food were determined exclusively on the basis of changes of weight and a clinical estimate of the exhaustion of the animal.

Conclusion: Under-nourished guinea-pigs in this series were able to stand injections of human serum or liver-suspensions from patients with hepatitis without having any temperature reaction; in some of the inoculated groups a rise of temperature was noted, which, however, was impressionable by small changes of diet. The experimental project made it impossible to decide whether some of temperature-increases were due to an infection, while others certainly coincided with changes of diet. Probably the injected animals are a little more inconstant as to body-temperature.

From the 16 animals mentioned above 7 liver-suspensions for inoculation into 14 new guinea-pigs were prepared. Later suspensions from their livers were inoculated in new animals and so on, the longest chain having 5 links. Altogether $16 + 68 = 84$ animals were used. The series originating from chronic hepatitis-material included $8 + 63$ animals (results from the first 2 passages indicated that the chronic series ought to be continued). — Fever was only found in 7 out of 68 animals.

Pathological examination of the 84 livers and microscopical examination of all but 10 showed no lesions corresponding to *Verlinde's* findings. Two livers were small, 6 large (as compared to the weight of the animal). An increased amount of glycogen was found by microscopy in 6 of these cases (and in 11 with normal liver-weight), while one of the small livers was normal, and one large liver was remarkably pale and fatty, but microscopically normal.

In eight cases many Kupffer cells were found, sometimes in connection with lymphocytes; besides two animals showed an increase in connective tissue. In 16 of the animals a more pale, but yellow-brown colour of the liver was found, but without connection to changes in weight or microscopy. (In five animals a localized macroscopic lesion of 2 to 4 mm's size was found, pink or white-coloured; microscopy only exists in some of these cases, showing ordinary abscess).

No connection was observed between the appearance of fever and the liver-lesions mentioned; males, females, and castrated females did not differ in these reactions.

Serological examinations: From the $16 + 68$ animals we obtained $14 + 56$ sera for serological purposes. 32 different liver-suspensions (12 of which were poolings) were prepared for antigen in complement-fixation. The antigens were taken from animals with fever or liver-lesions. Two antigens were found to react strongly with two sera, the titer being 1:300, and weakly with one (technique: see *Bjorneboe &*

Krag). The five animals from which these antigens or sera were taken resembled the others as to temperature and »liver-lesions«. — All these animals belonged to the chronic hepatitis series.

In all 1234 complement-fixation reactions were performed, all sera being tested against the two reacting antigens, and vice versa all the antigens were tested against the 3 reacting sera. Among the mentioned 32 antigens are also antigen from animals lying next to the 5 positive animals in the chain of infections.

Discussion.

By our experiments we have not been able to confirm the microscopic liver-changes described by Verlinde and van Genderen. On the other hand either complement-fixating antigen or antibody have been demonstrated by us in few of the animals injected with material from patients suffering from chronic hepatitis.

The experimental under-nutrition of our animals has hardly been of the same degree as that of Verlinde's animals; in contrast to Verlinde's series no deaths from under-nourishment alone occurred (among our animals were certain cases of death caused by intercurrent infection). Consequently the risk of liver-lesion caused by defective nutrition has been diminished, whereas on the other hand the chance of increased susceptibility to virus may have been less probable.

The temperatures of the guinea-pigs have been affected by additional food (carrots) given when the loss of weight was so considerable that the whole experimental scheme would be disturbed, as increase of temperature may follow shortly after addition of food.

The serological findings suggest the resumption of a similar experiment. In such an experiment should be proposed to keep the food absolutely uniform during the whole period (and resign to possible cases of death), and further make the serological examinations before a liver-suspension is used for injection in new animals to make it possible for the further passages of liver material to occur according to informations obtained from possible positive results.

Mac Callum's results of experiments (changes of tissue in lungs, lymph nodes, and liver) indicate that blind passages ought to be continued in 4—5 series.

It would be wrong to consider our results as a proof of the fact that it is impossible to transfer human hepatitis to guinea-pigs, but we admit our failure to prove the possibility of this.

Summary.

6 groups of under-nourished guinea-pigs (in all 28 animals) were injected with serum or liver-suspension from human cases of hepatitis; 4 of the 6 groups showed higher temperature from 2 to 4 weeks after

the injection. None of the animals showed microscopically signs of liver-degeneration as described by Verlinde and van Genderen.

The temperature in guinea-pigs seemed to follow the variations in nutrition, masking the interpretation of our results.

New groups of guinea-pigs were injected with liver-suspensions from animals in the 4 groups with fever — and so on taking 68 animals divided into 31 groups.

3 sera and 2 liver-suspensions showed together complement-fixation, more than 1000 other combinations were negative.

Experiments, allowing the positive serological findings to be instructing for the size of the individual series in the passages of the the suspected infection, should be undertaken.

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ON A COMMON ANTIGEN IN PROTEUS X 2 AND ESCHERICHIA COLI O GROUP 12

By F. Kauffmann and Beate Perch.

(Received for publication April 9th 1948.)

Continuing the investigations »Ueber Antigenbeziehungen zwischen Coli-, Dysenterie- und Salmonella-Bakterien« (1), we have widened their scope by including the Proteus group and in the following propose to describe an antigen that is common to Proteus X 2 and Escherichia coli O grup 12.

The O immune serum prepared from the original Proteus X 2 strain, which had a titre of 1:2560, agglutinated the test strain for Coli O group 12, viz. strain Bi 626/42, in a dilution of 1:1280. The same was the case with two other Proteus X 2 sera prepared with two Proteus X 2 strains isolated in Denmark and already published in a previous communication (2). It was there demonstrated that the Danish strains are identical with the original X 2 strain.

Another Coli strain of this group (H 501 a) behaved in the same manner as the test strain for Coli O group 12 (Bi 626/42); this strain was likewise strongly agglutinated in Proteus X 2 serum.

The O serum prepared with Coli strain Bi 626/42, which had a titre of 1:2560, agglutinated the Proteus X 2 strain at 1:640. This was also the case with another O serum of group 12 prepared by Knipschildt with strain H 501 a. Like the original Proteus X 2 strain, the other two Proteus X 2 strains agglutinated in both Coli sera of O group 12. These cross-reactions show that Proteus X 2 and Coli strains of O group 12 have a common O antigen.

When Proteus X 2 serum was absorbed by Coli strain Bi 626/42, only the agglutinins attacking the Coli bacteria were removed, whereas the Proteus agglutinins (titre 1:1280) remained in the serum. The corresponding action took place when Coli serum was absorbed by the Proteus X 2 strain: only the Proteus agglutinins were removed, whereas the Coli agglutinins remained in the serum, as will be seen from table 1.

Table 1.

Cultures	O Immunsera			
	Proteus X 2		Coli Bi 626/42	
	unabsorbed	absorbed by Coli Bi 626/42	unabsorbed	absorbed by Proteus X 2
Proteus X 2	2560	1280	640	0
Coli Bi 626/42	1280	0	2560	1280

These results show that the O antigens of the two cultures, *Proteus* X 2 and *Coli* Bi 626/42, contain not the same O antigen but merely have common partial antigens.

There is no O antigenic relationship between *Coli* O group 12 and *Proteus* X 19 or *Proteus* XK; thus the special position of the *Proteus* X 2 strain is clearly shown.

In order to determine whether *Coli* strains of O group 12 were agglutinated by sera from typhus patients, we have tested three human sera kindly placed at our disposal by the Institute of Hygiene of the Zürich University. Two of these sera (Belgrade 2 and Belgrade 4) agglutinated neither the *Proteus* X 2 strain nor the *Coli* 626/42 strain, whereas the third serum (Belgrade 11), while agglutinating *Proteus* X 19 and X 2, did not agglutinate 626/42 (not counting a slight agglutination in the serum, dilution 1:10, which might be of no significance; see Table 2).

Table 2.

100 °-culture	Typhus fever serum
<i>Proteus</i> X 19	1280
„ X 2	80
„ XK	<10
<i>Coli</i> Bi 626/42	10

Read after 20 hours in waterbath at 50° C.

It would appear from these experiments (Table 2) that in all probability there is no antigenic relationship between *Rickettsiae* and *Coli* strain Bi 626/42. But as this one patient serum agglutinated strain X 2 only slightly, no definite conclusion can be drawn. There is a possibility that other typhus sera with a higher X 2 titre would also agglutinate the *Coli* strain 626/42.

Discussion.

The O antigenic relationships demonstrated between *Proteus* X 2 and Coli O group 12 show that a positive Widal reaction with *Proteus* X 2 cannot always be attributed to a typhus infection, because in some cases it may be a Coli or *Proteus* X 2 infection. Coli O group 12 is one of the most frequent O groups (3) and therefore may give rise to an infection and a positive Widal reaction. The same applies to the *Proteus* X 2 strain, which is also ubiquitous and has recently been demonstrated by us in Denmark. Therefore, if we have to do with a positive X 2 Widal, we must include the Coli strain Bi 626/42 for control, in order to preclude at least a Coli infection.

Owing to the lack of suitable sera we were unable to determine whether there are antigenic relationships between *Rickettsiae*, the cause of typhus, and Coli strains of O group 12. A solitary test with a serum which agglutinated the X 2 strain at 1:80 was negative. In other words, the only O antigenic relationship is probably between *Proteus* X 2 and Coli strains of O group 12.

Summary.

A common O partial antigen in *Proteus* X 2 and strains of Coli O group 12 is reported. A positive X 2 Widal therefore does not always mean the presence of typhus, as it may also be due to an infection with *Proteus* X 2 strains or with strains of Coli O group 12.

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INFLUENCE OF FRACTIONED ROENTGEN RADIATION ON BACTERIAL AGGLUTINATION TITRE

By *Johannes Clemmesen and Else Krag Andersen.*

(Received for publication April 12th, 1948).

It is the aim of the present investigation further to examine the possibilities offered by Roentgen irradiation as a means for lowering resistance of experimental animals to inoculations of various kind.

Studies previously reviewed and reported have shown that universal exposure to Roentgen rays before inoculation will reduce resistance to homologous and heterologous tumour cells, (3, 6) to Shope fibroma virus (5) and to bacteria given per os (Chrom 1934). This reduction of resistance — which may be eliminated by parabiosis to a normal animal (Bichel & Holm-Jensen) — has been proved to consist in temporary delay of the development of immunity as far as Shope fibroma is concerned, and evidence has been produced that a similar process probably takes place at heterologous transfer of cells, whereas immunity to homologous cells may be completely suppressed (6), and — contrary to the others — broken even when once established.

However, on the whole it seems as if a single dose of rays, even if sublethal, is insufficient to influence other than weak immunities to a measureable extent and for more than a short period. Consequently, the first possibility for an increase of the said effect must consist in fractioned doses distributed over a longer period and thus probably less dangerous to the lives of the animals.

One of the difficulties expected might arise from the impossibility of influencing immunity by irradiation after the introduction of antigen. As has been said, most immunities, when once developed, are refractory to radiation, and preliminary experiments showed the same feature for the antigen here employed.

Technique: Irradiation was done by means of a »Siemens Roentgenbombe« for the use of which we are indebted to Dr. Jens Nielsen of The Radiumstation, Copenhagen. Conditions were the following: 193 kilowolts 20 milliamperes, 0,5 copper filtration, half-value layer 0,9 mm copper. As a rule the rabbits were exposed two at a time,

in a wooden box with a horizontal partition. The dimensions of the box were about $40 \times 30 \times 30$ cm. and the thickness of the walls 1 cm. In each subdivision the upper and lower half were connected with hinges on one side and two hooks on the other. In the end of the box a hole, fringed with leather would just close round the neck of a rabbit, thus keeping the animal quiet during irradiation.

Irradiation was given to the side of the animals with a distance from the focus to the skin of the rabbit of about 70 cm.

When half the dose had been given the box was turned so as to expose the other side of the animals. In this way homogeneous irradiation throughout the body was obtained. Measurements with a Siemens intensimeter at either side of a rabbit gave an intensity of 22 r and 6 r per minute respectively. Thus the intensity in the centre of the rabbit was estimated to 14 r per minute. The intensity immediately in front of the box was 23 r per minute.

Dosage was done by timing.

The effective dose was 120 r given five times, distributed as shown in the diagram.

Immunisation was carried out by a single intravenous injection of 0,1 cc. H-Antigen of *B. paratyphus B.* (formolized broth-culture). The antibody titre was determined daily by ordinary agglutination tests after incubation for 2 hours at 56° .

The diagrams give curves of the antibody titre, which have been slightly smoothed.

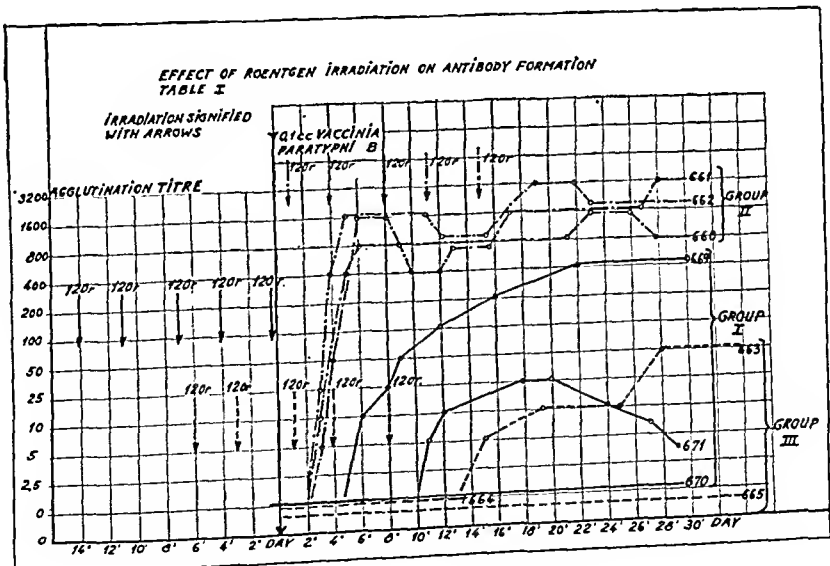
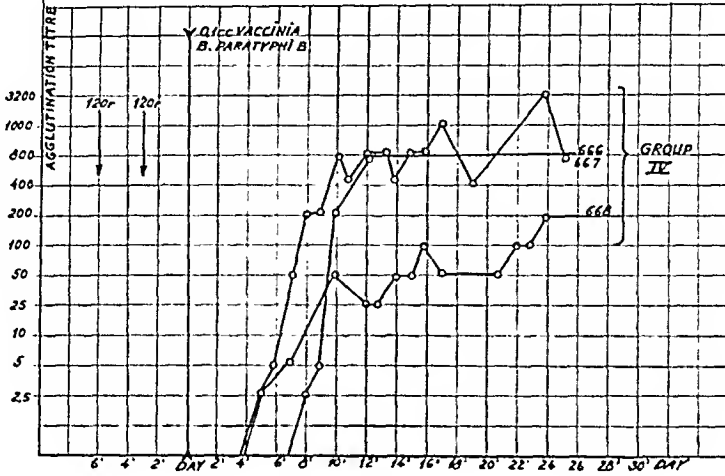
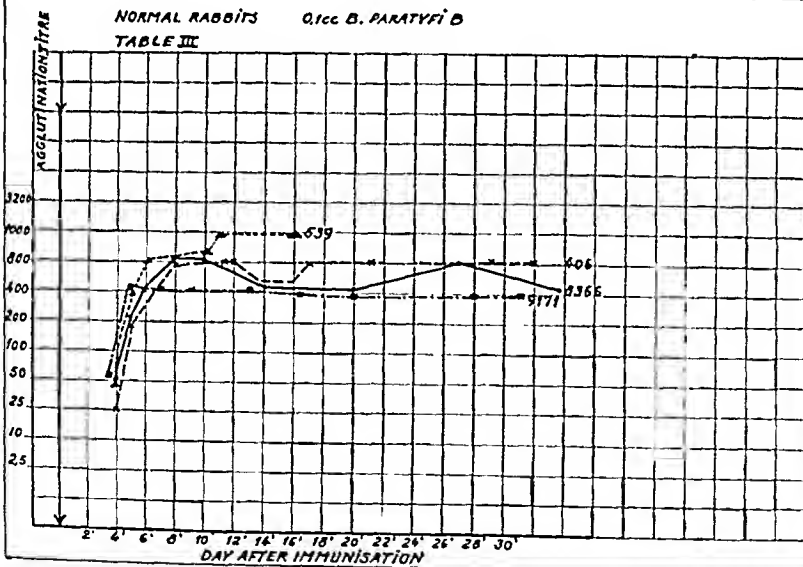


TABLE II



NORMAL RABBITS QICC B. PARATYPHI B
TABLE III



After preliminary examinations we performed the experiment described on tables I and II: Four groups, each of three rabbits, were all immunised and irradiated with the same doses. Group I received its full dose of rays before immunisation, group II the same dose after immunisation. Group III received a small ($120 \text{ r} \times 2$) dose before immunisation and the rest afterwards, while a fourth group only had this light pre-irradiation of $120 \text{ r} \times 2$, and behaved almost like a set of normal controls given on table III. (It may be added that only 1 out of 8 rabbits which in other experiments received a treatment similar to group III developed an agglutination titre of 1:25).

Conclusions:

From the resulting curves on the agglutination titre and our general experience it seems justifiable to conclude:

1. Fractioned doses are more effective for lowering of resistance than single doses.

2. It seems possible to prevent development of immunity without serious damage to the general health of the animals.

3. A dose of rays which if given before immunisation (gr. I) will delay the development of immunity, does not show the same effect if applied after immunisation (gr. II).

4. If an, in itself insufficient, part of the radiation has been applied before immunisation (gr. III), the remaining dose given after the latter will be more effective than the total dose given at the same period. Even an immunity otherwise apparently refractory to radiation may in this way be prevented from developing.

5. The effect may be visualized as a blockage of the R. E. S. which must be established at the appearance of antigen and must be repeatedly renewed in the presence of the latter.

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INFLUENCE OF ROENTGEN RADIATION ON THE SPREAD OF VACCINIA VIRUS IN YOUNG RABBITS

By *Johannes Clemmesen and Else Krag Andersen.*

(Received for publication April 12th, 1948).

Continuing the investigations described in the preceeding paper, we have in the following examined if the effect of Roentgen radiation on immunisation could be exploited in making rabbits sensitive to vaccinia virus.

As we wished to create the most favourable conditions for the said effect we used for the experiment very young rabbits, which have a weaker response to antigens than older animals, presumably due to a less developed reticulo-endothelial system.

The irradiation was applied according to our earlier experience with allowance for the young age of the animals. As we have not been able to carry out determination of lethal doses on young animals we may in the first experiment have used too small doses, (540 r) although the last dose given was very heavy, because the time necessary to the lethal effect was unlikely to expire before the killing of the animals. In the second experiment, on quite young animals, the dose used was 420 r and seems to have killed two of the animals, though perhaps in connection with the virus infection.

Technique.

Roentgen radiation was applied with a »Siemens Roentgenbombe« under the following conditions:

Experiment 1: 200 kilovolts, 30 milliamperes, 0.5 mm copper filtration.

Experiment 2: 193 kilovolts, 20 milliamperes, 0.5 mm copper filtration, halfvalue layer: 0.9 mm copper.

The young rabbits were exposed two or more at a time, in a wooden box designed for irradiation of adult animals. The wall measured 1 cm. in thickness. Irradiation was given at a distance of ca. 70 cm. from the focus to the skin of the animals. When half the dose had been given, the box was turned so as to expose the other side of the animals. In this way we tried to obtain homogenous irradiation

throughout the body. Dosage was done by timing, and the intensity in Exp. 1 was about 21 r per minute, in Exp. 2 about 16 r per minute at the distance stated above. This — presumably insignificant — change in the conditions was caused by war difficulties. Provided the absorption in the wall of the box is negligible, and that measurements carried out with adult rabbits hold good in this case, the effective average dose given throughout the animals amounts to about 61 per

Experiment 4.

9 Rabbits, two months old — weight 1000—1100 gr.

6 Rabbits irradiated.

3 Controls.

12 days before inoculation 120 r eff.

8 days — — 60 r

5 days — — 60 r

1 day — — 120 r

Vaccinia 0,1 cc X 6 intradermally to all animals.

2' day after inoculation: 180 r.

3' day after inoculation: 2 exp. animals and 1 control killed

		Blood	Liver	Spleen	Lymph node
Titration of virus	X 1	0	0	0	1:10
	X 2	0	0	0	1:10
	Control 1	0	0	0	1:10

6' day after inoculation: 2 exp. animals and 1 control killed

Titration of virus	X 3	0	0	0	1:10?
	X 4	0	0	0	0
	Control 2	0	0	0	0

9' day after inoculation: 2 exp. animals and 1 control killed

Titration of virus*).....	X 5	1:20	1:10	1:10	1:10
	X 6	0	0	0	0
	Control 3	0	0	0	1:10

*) The patho-anatomical picture was not typical of a generalised spread of virus: Lymph nodes were enlarged but liver and spleen were normal. No spotted appearance of kidney.

Experiment 2.

13 rabbits. Weight about 250 gr. Age 35 and 33 days at inoculation.

Two litters respectively of 7 and 6 rabbits were equally distributed on experimental animals and controls.

— 12 days before inoculation 60 r

— 8 days — — 60 r

— 5 days — — 60 r

— 2 days — — 60 r

Vaccinia 0.1 cc. X 6 intradermally.

1' day after inoculation: 180 r.

3' day after inoculation: 2 exp. animals and 1 control killed.

		Blood	Liver	Spleen	Lymph node
Titration of virus	X 1	0	0	0	1:10
	X 2				
Control	1	0	0	1:10	1:10

5' day after inoculation: 2 exp. animals and 1 control killed

Titration of virus	X 3	10 ⁻²	1:10	1:10	>10 ⁻⁶
	X 4	10 ⁻²	1:10	10 ⁻²	>10 ⁻⁶
Control	2	0	0	1:10	1:10

6' day after inocul. died .. X 9

10⁻⁵ 0 10⁻⁴

7' day after inoculation: 2 exp. animals and 1 control killed

Titration of virus	X 5	1:10	10 ⁻³	0?	10 ⁻⁶
	X 6	0?	1:10	0	0
Control	3	0	0	0	0

8' day after inocul. died .. X 8

10⁻⁵ 0 10⁻⁴

9' day after inoculation: 1 exp. animal and 1 control killed

Titration of virus	X 7	10 ⁻²	0	0	10 ⁻⁴
Control	4	0	0	0	0

cent of the skin dose, if half the dose is applied to each side of the animal.

Vaccinia (Bl 1/43, 1 + 1) was injected intradermally in six places each with a dose of 0.1 cc, into each of the young rabbits, which were killed at intervals of a few days. Regional lymph nodes and various organs were ground with saline and injected intradermally in an adult rabbit in various dilutions according to the usual technique for titration.

Conclusions:

Heavy Roentgen irradiation with fractioned doses failed to show any certain effect favouring the spread of vaccinia virus in rabbits two months old. There may be signs of a weak effect, and it cannot be excluded that the dosage may have been insufficient.

A corresponding experiment performed on young rabbits about 30 days old, with sublethal doses of Roentgen rays showed a definite spread of vaccinia in most experimental animals, but none in the controls.

SUPPLEMENT TO THE PAPER »ZUR SEROLOGIE DER DYSENTERIE-GRUPPE«

By F. Kauffmann.

(Received for publication may 8th, 1948).

It is our purpose in the present paper to correct an error in the nomenclature of the cultures employed in an earlier work, »Zur Serologie der Dysenterie-Gruppe«.

It should be explained that, unfortunately, in the paper cited above the work of Boyd was not considered, since it was not available to the author during wartime. The papers of Wheeler and Weil on the serology of the Shigella group appeared after our publication, so comparative investigations of the cultures used could not be carried out until recently. Ferguson, Weil and Wheeler examined my cultures and I am indebted to these authors for having placed test strains at my disposal. Our investigations are in complete agreement concerning the identity of the strains employed earlier by me.

It was discovered that the strain designated »Oxford W« by the author was in reality an »Oxford V« strain, therefore, in the paper »Zur Serologie der Dysenterie-Gruppe«, one must substitute the designation »Oxford V« for »Oxford W«. The cause of this incorrect designation was a writing error which occurred at some time during the transfer of cultures in the collection belonging to the State Serum Institute.

In order that the designations of the remaining strains may be compared with those of other authors, they have been recorded in tabular form as follows:

Table 1.

Kauffmann	Nomenclature of		
	Andrewes and Inman	Wheeler	Weil
Oxford W*)	V	Ib	I
Stansfield VZ	VZ	Ia	I. III
A 531 Kruse	VZ	Ia	I. III
D 118 Kruse	W	IIa	II
Mountain WX	WX	IIb	II. VII
H 39 Kruse	Z	III	III
Whittington Z	Z	III	III

*) The designation »W« was a writing-error and must be substituted by »V«.

It will be seen from table 1 that the cultures used by the author comprised 5 Flexner types. The antigenic formulae of these types are stated in table 2.

Table 2.
Antigenic Structure of 5 Flexner Types.

Nomenclature of Wheeler	Antigens according to Kauffmann
Ia	1, 2, 4, 5, 7
Ib	1, 4, 5, 7
IIa	1, 3, 5, 8
IIb	1, 3, 4, 6
III	1, 2, 4, 6

It should be made clear that the purpose of the above-cited paper was not to set up complete antigenic formulae for the Flexner types but to record the diagnostically important antigenic relationships. It was shown that by means of exactly defined factor sera the differential diagnosis of the 5 types tabulated in table 1 was accomplished without effecting a distinction in »type« and »group« antigens. The most important results of these investigations, it was pointed out, was that distinct *qualitative* antigenic differences occur between these 5 Flexner types; therefore it is possible to make the type diagnosis by means of factor sera in a slide agglutination test.

It was also demonstrated by a comparison of strains that our culture designated »Hiss-Russell Y« belonged to type II a (= W = D).

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MEMORANDUM ON THE FLEXNER GROUP

by F. Kauffmann and W. W. Ferguson.

(Received for publication May 8th 1948.)

At the present a variety of nomenclature is in use for the entities of the Flexner group. This varies from the race and type designations of Andrewes and Inman and Boyd, respectively, to the designation of Wheeler and Weil. It is proposed that a new schema be adopted which is in agreement with bacteriological nomenclature.

If we agree that the ultimate designation in bacteriological nomenclature is the type, it becomes apparent that the term »type« for Flexner organisms I, II and IV is a misnomer. These entities are really subgroups of the Flexner group and each consists of two different types. The following schema designates the Flexner types in a true sense, according to present-day knowledge:

Shigella flexneri

Proposed Designations

Type 1a }
Type 1b } simplified: type 1 (subgroup 1)

Type 2a }
Type 2b } simplified: type 2 (subgroup 2)

Type 3

Type 4a }
Type 4b } simplified: type 4 (subgroup 4)

Type 5

Type 6

The typing of Flexner organisms as accomplished by absorbed sera prepared after the method of Boyd and Wheeler is a simplified diagnostic practice. In reality it is partly a subgroup determination and partly a type determination. There is no question that it is possible

to demonstrate in antisera for Flexner strains V (1), W (2), Z (3), 103 (4), P 119 (5) and 88 (6) the existence of agglutinins specific for the homologous subgroup or type. But in subgroup 1, Type 1b (V) is clearly different from Type 1a (VZ or I, III). The latter contains an antigen that is not found in Type 1b and differentiates 1a from 1b; furthermore, this antigen is not found in any of the other types and can therefore not be regarded as a »group antigen«. In subgroup 2, Type 2a (W) is different antigenically from 2b (WX). The difference or specificity in this case lies in the »group antigen«, not in the »type specific«, as both types have quite different »group antigens«. In subgroup 4, Type 4a is different antigenically from 4b, as both types have different »group antigens« (according to Wheeler and Ferguson).

For the determination of the subgroups 1, 2 and 4 and the types 3, 5 and 6, we must prepare factor sera I to VI, and for the determination of the types in subgroups 1, 2 and 4, additional factor sera (Ia, IIa, IIb, IVa or IVb). The type diagnosis in the subgroups is made in two steps:

1. determination of subgroups and
2. determination of types.

The proposed schema is sufficiently elastic that new types may be fitted into it without difficulty. It seems probable that new types — that is, new combinations of the so-called »type antigens« and »group antigens« — will be discovered if identification of Flexner organisms is generally carried to the point of type determination.

Flexner X and Y are not recognized as independent types, but as loss variants only.

The designation »double types« should no longer be used, as these organisms are only »types« (1a and 2b, respectively).

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ÜBER DIE KONSTANZ UND VERERBUNG DER SEROLOGISCHEN QUANTITÄTEN (IM LICHT DES ISOAGGLUTINATIONSPHÄNOMENS)¹⁾

Von Prof. Dr. *Oskari Renkonen* (Streng).

(Eingegangen bei der Redaktion am 19. Februar 1948.)

Die serologischen Quantitäten, ihre Konstanz und Vererbung, ihre Abhängigkeit von Alter, Geschlecht, Gesundheitszustand usw. wurden in besonders grosser Ausdehnung und in mehreren Richtungen im Anschluss an die Organisation des Blutspenderdienstes in Finnland während der Kriegsjahre untersucht. Unsere Resultate betreffs der Variationen sowohl der Titerstärke der isoagglutinierenden Sera als der Empfindlichkeit der Blutkörperchen werden in nächster Zukunft veröffentlicht. In diesem Vortrage wird nur in aller Kürze über einige bei uns gemachte Erfahrungen betreffs der Theorie des Isoagglutinationsphänomens berichtet.

Zuerst seien einige von mir hauptsächlich zusammen mit Dr. *Esko Suomalainen* gesammelte Zahlen, Material I, betreffs der Konstanz der Isoagglutinationstiter erwähnt. Die 2 %-Aufschwemmungen der frischen steril entnommenen und von 0 bis paar Tage in Eisschrank aufbewahrten Blutkörperchen und ebenso die Serumverdünnungen wurden in der Menge von 0,1 cm³ benutzt. Die Verdünnungsart geht aus der Tabelle I hervor. Die Reaktionszeit war 1 Stunde bei 37° und ½ bis 1 Stunde bei Zimmertemperatur. Die Ablesungen der Resultate wurden makroskopisch nach Aufschütteln möglichst analoger Art durchgeführt. Als Blutkörperchenspender für die Reaktionen fungierten in der A₁-Gruppe haupt sächlich 4 Personen, in der A₂-Gruppe 2 und in der B-Gruppe 2 Personen. Die Resultate gehen aus der Tabelle I hervor, welche die Frequenz der verschiedenen starken Reaktionen angibt.

1) Nach einem im Juli 1947 in Uppsala auf dem Nordischen Pathol. Kongress gehaltenen Vortrag. Der Vortrag war ganz kurz (hauptsächlich nur Tabellen aus welchen einige Rechnungsfehler hier korrigiert worden sind) und wird jetzt in ausführlicherer Form vorgelegt.

Tabelle I.)
Material I.

Die Verdünnungen	2/1	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Summe
O-Serum A ₁ -Blutk.												
Anzahl		3	19	220	816	2732	1794	1194	267	54	2	7101
%		0.04	0.3	3.1	11.5	38.5	25.2	16.8	3.8	0.8	0.02	
B-Serum A ₁ -Blutk.												
Anzahl	—	—	6	46	123	417	252	159	44			1047
%			0.6	4.4	11.7	39.8	24.1	15.2	4.2			
Anti-A ₁ ins Gesamt.												
Anzahl		3	25	266	939	3149	2046	1353	311	54	2	8148
%		0.03	0.3	3.3	11.5	38.7	25.1	16.6	3.8	0.7	0.02	
O-Serum B-Blutk.												
%	0.4	0.7	1.9	15.0	25.9	36.0	13.9	5.2	0.9	0.1		
Anzahl	27	47	129	1023	1772	2457	950	358	60	5	—	6828
A-Serum B-Blutk.												
Anzahl			29	307	602	1038	336	140	20	2		2474
%			1.2	12.4	24.3	41.9	13.6	5.7	0.8	0.1		
Anti-B, ins Gesamt.												
Anzahl	27	47	158	1330	2374	3495	1286	498	80	7		9302
%	0.3	0.5	1.7	14.3	25.5	37.6	13.8	5.3	0.9	0.1		
O-Serum A ₂ -Blutk.												
Anzahl		11	6	61	76	117	14	8	2			295
%		3.7	2.0	20.7	25.8	39.7	4.7	2.7	0.7			

Die Tabelle zeigt, dass die gewöhnlichste Frequenz der Reaktionsstärke betreffs Anti-A₁ bei der Verdünnung von 1/16 bis 1/32 liegt, während dieselbe betreffs Anti B bei 1/8—1/16 liegt.

Aus der Tabelle ist somit dasselbe ersichtlich, was schon *Thomsen* und *Kettel* in Dänemark gefunden haben, nämlich dass die Titerwerte für Anti-A₁ sowohl in O- als in B-Sera und ebenso die Werte für Anti-B sowohl in O- als in A-Sera einander folgen. Die Werte für Anti-A₁ sind grösser als die Werte für Anti B. Wie die ausgerechneten Mittelwerte für Anti A₁-, Anti A₂- und Anti-B-Reaktionen sich in O-Sera verhielten, geht aus der Tabelle II hervor.

Aus der Tabelle II ist zu ersehen, dass die Mittelwerte der Reaktionen in jeder Gruppe, trotzdem die als Massstab benutzten Blutkörperchen von verschiedenen Personen stammten, unter sich ganz ähnlich waren. Dasselbe konnte auch in A- und B-Sera konstatiert werden.

Eine Vorbedingung ist, dass die Anzahl der bei der Mittelwertberechnung berücksichtigten Sera genügend gross ist. Wenn die Mittel-

1) Die Arbeit mit Dr. E. Suomalainen wird ausführlich in Annal. med. exper. et biol. Fenniae erscheinen.

Tabelle II.
Material I.

Mittelwerte der Titerwerte.			
O Anti-A ₁ .			
	Mittelwerte	Anzahl	
Blut von Fräul. H	31.27	2	468
» K—a	35.20	1	743
» N—i	37.42	1	354
» K—n	30.82		56
Herren U	27.49		689
NN	30.89		791
	32.95	7	101
O Anti-B.			
Blut von Fräul. K—mo	18.21	3	080
» S—n	16.59	2	976
» S—i			13
» S—d			8
N. N.	18.97		751
	17.60	6	828
O Anti-A ₂			
Blut von N:o 4307	14.93		167
» 471	6.75		44
» 4300			22
» 2280			1
N. N.	12.66		61
	13.43		295

werte aus Gruppen von nur 100 Sera berechnet werden, können die Mittelwerte um eine ganze Stufe variieren. In Gruppen von mehreren Hunderten waren die Variationen der Mittelwerte bedeutend kleiner.

Um zu sehen, wie grosse eventuelle Fehler bei unserer Technik und Ablesung vorkommen können, haben wir mehrmals an verschiedenen Tagen dieselben Sera mit Blutkörperchen derselben Personen untersucht und dabei eine gute Übereinstimmung der Resultate gefunden. Nur ganz vereinzelt war eine Differenz von 2 Titerstufen vorhanden, meistens waren die Resultate identisch oder waren Differenzen vom $\frac{1}{2}$ bis 1 Stufe vorhanden. Differenzen von $1\frac{1}{2}$ Stufen waren selten.

Trotzdem unsere in der Tabelle II wiedergegebenen Resultate betreffs der Mittelwerte einheitlich waren, kann man natürlich nicht auf Grund derselben von einer absoluten Konstanz der Mittelwerte der Reaktionen sprechen. Wenn eine andere Reaktionsstärke, als die von uns als Grenzwert verwendete, benutzt worden wäre, hätten sich natürlich auch die Mittelwerte verschoben. Die Bedeutung einer Veränderung des Grenzwertes bei der Ablesung und bei öfterem Wechseln der Blutkörperchen spendende Personen geht aus der Tabelle III hervor.

Tabelle III.
Material II.

Titerstärke	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	Summe
O-Serum A-Blutk.											
Anzahl	—	1	10	21	57	81	97	71	3	3	344
%		0.3	2.9	6.1	16.6	23.5	28.2	20.6	0.9	0.9	
B-Serum A-Blutk.											
Anzahl	—	26	24	38	78	106	129	52	4	1	458
%		5.6	5.2	8.3	17.0	23.2	28.2	11.4	0.9	0.2	
O-Serum B-Blutk.											
Anzahl	1	2	20	28	42	50	46	10	—	—	199
%	0.5	1.0	10.1	14.1	21.1	25.1	23.1	5.0			
A-Serum B-Blutk.											
Anzahl		16	54	77	118	103	74	32	1	—	475
%		3.4	11.4	16.2	24.8	21.7	15.6	6.7	0.2		

Die in dieser Tabelle angegebenen, von mir später gesammelten Zahlen, Material II, sind aus Serien erhalten, die zwar unter sich technisch gleichartig behandelt, aber sonst unter sich möglichst verschiedenartig waren. Es wurde ein anderer Grenzwert bei der Ablesung gewählt als bei den in Tabelle I und II referierten. Weiter wurden jeden Tag Sera und frische Blutkörperchen von neuen, früher nicht untersuchten Personen genommen. Während in der Tabelle I (Material I) der gewöhnlichst vorkommende Reaktionstiter für Anti-A einer Serumverdünnung von 1 : 16 entsprach, war der entsprechende Titerwert in Tabelle III (Material II) 1 : 64. Für Anti-B waren die entsprechenden Zahlen in der Tabelle I 1 : 16 und in Tabelle III 1 : 32.

Tabelle IV.

Verhältnis der Mittelwerte, der Titerstärke und der Frequenzahlen.

Material I Mittelwerte I	Material II Mittelwerte II	Frequenzahlen
$\frac{0 \text{ Anti-A}_1}{0 \text{ Anti-B}} = \frac{32.95}{17.60} = 1.87$	$\frac{61.96}{34.17} = 1.81$	$\frac{A_1}{B} = 1.9$
$\frac{B \text{ Anti-A}_1}{A \text{ Anti-B}} = \frac{30.30}{18.39} = 1.65$	$\frac{47.03}{31.87} = 1.48$	$p/q = 1.6$
$\frac{0 \text{ Anti-A}_1}{0 \text{ Anti-A}_2} = \frac{32.95}{13.43} = 2.40$		$\frac{A_1}{A_2} = 2.8$
$\frac{0 \text{ Anti-A}_2}{0 \text{ Anti-B}} = \frac{13.43}{17.60} = 0.76$		$\frac{A_2}{B} = 0.7$
In Dänemark (Kettel u. Thomsen*)		
$\frac{0 \text{ Anti-A}_1}{0 \text{ Anti-B}} = \frac{332}{112} = 3.0$		$\frac{A_1}{B} = 3.2$

*) von mir ausgerechnet.

Aus dem ganzen Material I (Tabelle IV) wurde für Anti-A, ein Mittelwert der Reaktionsstärke von 1 : 32,95 gefunden. In dem Material II (Tabelle IV) war der entsprechende Mittelwert 1 : 61,96. Für Anti-B waren die Zahlen 1 : 17,6 resp. 1 : 34,17 usw. Also nicht dieselben konstanten Zahlen in beiden Materialien. Die nach veränderter Ablesung des Grenzwertes gefundenen Titerwerte hatten sich aber unabhängig davon, welche Blutart A₁ oder B untersucht wurden, gleichförmig verschoben. Das Verhältnis der Mittelwerte $\frac{0 \text{ Anti-A}}{0 \text{ Anti-B}}$, $\frac{B \text{ Anti-A}}{A \text{ Anti-B}}$ usw. war in beiden Materialien heinahe dasselbe. Somit könnte man vielleicht von einer Konstanz der Verhältnisse der Mittelwerte z. B. $\frac{0 \text{ Anti-A}}{0 \text{ Anti-B}}$ usw. sprechen, obwohl die ursprünglichen Werte 0 Anti-A, 0 Anti-B usw. je nach der Ablesung der Grenzwerte und der Art des Materials im übrigen verschieden war.

Wenn sowohl die Stärke der Sera als auch die Empfindlichkeit der Blutkörperchen beides bei den Individuen in einer Population variierende Grössen wären, würde man dennoch nicht von einem konstanten Werte der obengenannten Verhältniszahlen in absoluter Bedeutung sprechen können. Eine Vorbedingung hierzu wäre, dass entweder die Sera der Erwachsenen oder die Empfindlichkeit der Blutkörperchen in einer Population innerhalb der Typengruppen unverändert dieselben Werte haben. Betreffs des Titerwertes der Sera ist dieses wie bekannt nicht der Fall. Die Werte der einzelnen Sera variieren auch in dem Falle, dass dieselben Blutkörperchen als Massstab benutzt werden. Tabelle I. Betreffs der Empfindlichkeit der Blutkörperchen der Erwachsenen in einer Population haben *Thomsen* und *Kettel* zu der Zeit, als die Viergenentheorie noch nicht klargestellt war, gefunden, dass man in 85 % bei der Arbeit mit Blutkörperchen der verschiedensten Provenienz dieselben Titerwerte der Sera findet. Unsere Versuche sprechen dafür, dass frisch entnommene Blutkörperchen verschiedener erwachsenen Individuen innerhalb der einzelnen Typengruppen, also in der A₁- und B-Gruppe, in einer Population, betreffs der Empfindlichkeit bei der Isoagglutination meistens identisch sind. Die Unterschiede sind meistens kleine doch kommen auch grössere Unterschiede obwohl mehr selten vor. Dieses ist aber nur gültig unter der Bedingung, dass die *lege artis* aufbewahrten Blutkörperchen von erwachsenen Individuen stammen und möglichst früh nach der Entnahme untersucht werden. Die Blutkörperchen der Kinder werden nämlich bis 20 J. immer empfindlicher (*Kemp*). Mit steigendem Alter werden zwar auch die Reaktionen allmählich schwächer, *Thomsen* u. *Kettel*. Dieses haben auch wir gefunden. Teoretisch könnte man sich denken, dass die Abschwächung der Reaktionsstärke mit dem Alter von einer Abschwächung der Sera oder einer Abstumpfung der Empfindlichkeit der Blutkörperchen abhängig wäre. Die letztgenannte Möglichkeit ist nicht vorhanden (*Thomsen*), wohl aber die erstgenannte,

und werde ich einer später erscheinenden Arbeit ausführlicher über unsere Erfahrungen betreffs dieser Frage berichten.

Weil die Frage, ob die Empfindlichkeit der Blutkörperchen verschiedener Provenienz innerhalb der Typengruppen in einer Population identisch oder verschieden ist, wichtig und noch nicht als endgültig aufgeklärt zu betrachten ist, habe ich, um die Frage zu beleuchten, verschiedene Materialien in folgender Art näher behandelt.

Das früher erwähnte Material II z. B. war, wie gesagt, so gesammelt, dass alle Tage neues Blut von neuen Individuen austitriert wurde. Alle Sera der A-Personen wurden mit allen B-Blutkörperchen desselben Tages austitriert und ebenso die B-Sera mit allen A-Blutkörperchen, d. h. alle Blutarten desselben Tages wurden kreuzweise miteinander austitriert. Alle AB-Blutkörperchen wurden mit A- und B-Sera untersucht, alle O-Sera mit A- und B-Blut (Tabelle III). Alle Tage hatte ich weiter zur Verfügung mehrere A-Sera, die mit B-Blut derselben Individuen austitriert worden waren, und ebenso mehrere A-Blutkörperchen, deren Empfindlichkeit mit B-Sera derselben Person untersucht worden waren. Weiter hatte ich auch verschiedene B-Sera, die am selben Tage mit denselben A-Blutkörperchen, und verschiedene B-Blutkörperchen, die mit denselben A-Sera untersucht worden waren. Ebenso standen mir zur Verfügung mehrere O-Sera, welche mit denselben A- und B-Blutkörperchen austitriert wurden und B-Blutkörperchen, die mit denselben O-Sera austitriert waren. Ebenso wurden die AB-Blutkörperchen mit denselben A- und B-Sera austitriert. (Siehe das Schema für O-Sera.)

Nur die Reaktionsresultate, welche am selben Tage mit verschiedenen O-Sera und mit demselben B-Blut erhalten waren, wurden als Paare miteinander verglichen. Ebenso wurden die mit verschiedenen O-Sera und demselben A-Blut am selben Tage erhaltenen Resultate als Paare verglichen. Ebenso wurden weiter die am selben Tage mit denselben O-Sera und verschiedenen A- resp. B-Blutkörperchen erhaltenen Reaktionen als Paare gegenüber einander gestellt usw. Die Reaktionen in horizontaler Richtung in dem Schema I geben die Unterschiede der Paare betreffs der Empfindlichkeit der Blutkörperchen an, diejenigen in vertikaler Richtung die Unterschiede der Paare betreffs der Serumstärke. Die an verschiedenen Tagen erhaltenen Unterschiede der Paare wurden zusammengezählt. Die Resultate mit O-Sera gehen aus der Tabelle V hervor. Die Resultate mit A- und B-Sera, Tabelle VI, waren diesen analog. Am selben Tage wurden nur 2 bis 5 verschiedene A-Sera mit 2 bis 5 verschiedene B-Blutkörperchen und umgekehrt 2 bis 5 B-Sera mit 2 bis 5 A-Blutkörperchen austitriert. Die am selben Tage untersuchten A-Sera und A-Blutkörperchen resp. B-Sera und B-Blutkörperchen stammten von denselben A- resp. B. Individuen. Die O-Sera wurden mit denselben A- resp. B-Blutkörperchen mit welchen die B- resp. A-Sera austitriert wurden, untersucht. Die Empfindlichkeit der AB-Blutkörperchen wurde mit denselben A- resp. B-Sera, mit welchen die Empfindlichkeit der A- resp. B-Blutkörperchen festgestellt war, bestimmt.

Schema I.)

B-Blutkörperchen I.							B-Blutkörperchen II.							B-Blutkörperchen III usw.						
Die Titerstärke.							Die Titerstärke.							Die Titerstärke.						
$1/4$	$1/2$	$1/4$	$1/8$	$1/16$	$1/32$	usw.	$1/4$	$1/2$	$1/4$	$1/8$	$1/16$	$1/32$	usw.	$1/4$	$1/2$	$1/4$	$1/8$	$1/16$	$1/32$	usw.
O-Serum I	+	+	+	—	—		+	+	+	+	—	—		+	+	+	+	—	—	
» II	+	+	—	—	—		+	+	+	—	—	—		+	+	—	—	—	—	
» III	+	+	+	+	—		+	+	+	+	—	—		+	+	+	+	—	—	
						usw.							usw.							usw.
A-Blutkörperchen I.							A-Blutkörperchen II.							A-Blutkörperchen III usw.						
Die Titerstärke.							Die Titerstärke.							Die Titerstärke.						
$1/4$	$1/2$	$1/4$	$1/8$	$1/16$	$1/32$	usw.	$1/4$	$1/2$	$1/4$	$1/8$	$1/16$	$1/32$	usw.	$1/4$	$1/2$	$1/4$	$1/8$	$1/16$	$1/32$	usw.
O-Serum I	+	+	+	—	—		+	+	+	+	—	—		+	+	+	+	—	—	
» II	+	+	—	—	—		+	+	+	—	—	—		+	+	+	—	—	—	
» III	+	+	+	—	—		+	+	+	+	—	—		+	+	+	+	—	—	
						usw.							usw.							usw.

*) Die in dem Schema angegebenen Titerwerte sind schematisch und entsprechen nicht gefundenen Werte. In Tabelle III ist die Anzahl der Endwerte der verschiedenen Titrationen und nicht die Anzahl der Personen angegeben. In den Tabellen V und VI sind die Differenzen unter den am selben Tage erhaltenen Titerwerte der Paarlinge in Stufen angegeben.

Tabelle V.*)
Material II.

Material II.														
O Anti-B.														
Differenzen in Stufen.														
222 Paare (verschied. Serum, dieselben Blutk.).													Die Anzahl der Reaktions- paare = 222	
0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	5 1/2	6		
29	25	51	14	45	8	35	3	8	2	2				
105			117											
47,3 %			52,7 %											
195 Paare (dasselbe Serum, verschied. Blutk.).													= 195	
76	47	56	5	9	1	1								
179			16											
91,8 %			8,2 %											
O Anti-A.														
383 Paare (verschied. Serum, dieselben Blutk.).														
67	59	89	35	54	16	30	6	14	4	6	2	1	= 383	
215			168											
56,1 %			43,9 %											
580 Paare (dasselbe Serum, verschied. Blutk.).														
177	143	140	50	49	9	10	—	2					= 580	
460			120											
79,3 %			20,7 %											

Aus den Tabellen V und VI geht deutlich hervor, dass die Empfindlichkeit der Blutkörperchen der Erwachsenen unter sich viel weniger differieren als die Titerwerte der Sera. Die B-Blutkörperchen waren, mit O-Serum gemessen, in 91,8 % einander ähnlich. Wenn auch Unterschiede von 1 1/2 Stufen noch als innerhalb der technischen Fehler liegend betrachtet werden könnten, wäre die Übereinstimmung noch grösser. Mit A-Sera gemessen war die Ähnlichkeit der B-Blutkörperchen unter sich auch gross. Wenn Unterschiede von 1 1/2 Stufen noch als innerhalb der technischen Fehlergrenzen liegend betrachtet werden können, würde die Ähnlichkeit auch hier bis über 90 % steigen. Die Tatsache, dass die Unterschiede betreffs der Empfindlichkeit der A-Blutkörperchen im Material II nicht so klein sind wie betreffs der B-Blutkörperchen, liegt offenbar darin, dass in diesem Material die A₁- und A₂-Blutkörperchen nicht auseinandergehalten worden waren. Wenn die A₁- und A₂-Blutkörperchen als zwei Gruppen jedes für sich untersucht worden wären, hätte man wahrscheinlich auch hier dieselbe Ähnlichkeit der Empfindlichkeit wie für B-Blut gefunden. Dieses zeigen später ausgeführte Untersuchungen betreffs der Heredität der Empfindlichkeit der Blutkörperchen, welche später referiert werden sollen.

Obwohl somit dann und wann ganz abweichende Unterschiede zwi-

Tabelle VI.')

Material II.

A Anti-B.													Die Anzahl der Reaktions- paare
957 Paare (verschied. Serum, dieselben Blutk.).													
Die Differenzen in Stufen.													
0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	5 1/2	6	= 957
127	150	234	137	135	53	65	22	22	5	7	—	—	
511			446										
53.4 %			46.6 %										
420 Paare (dasselbe Serum, verschied. Blutk.).													= 420
126	106	117	42	14	8	1	1	3	1	1	—	—	
349			71										
83.2 %			16.8 %										
B Anti-A.													520
520 Paare (verschied. Serum, dieselben Blutk.).													
92	72	127	59	64	33	37	13	12	1	5	1	4	
291			229										
54.0 %			46.0 %										
583 Paare (dasselbe Serum, verschied. Blutk.).													= 583
112	121	167	61	63	30	20	6	2	1	—	—	—	
400			183										
68.6 %			31.4 %										

schen der Empfindlichkeit der Blutkörperchen von uns notiert worden sind, scheint dieses doch praktisch genommen für die Frage der Konstanz der Verhältniszahlen des Mittelwertes $\frac{0 \text{ Anti-A}}{0 \text{ Anti-B}}$ usw. von untergeordneter Bedeutung zu sein. Die Hauptsache ist, dass die Stärke der individuellen Sera in einer Population unter sich sehr verschieden ist, während die verschiedene Empfindlichkeit der Blutkörperchen nur eine untergeordnete Bedeutung für die Grösse der Titerwerte hat. Vergleiche die entgegengesetzten Resultate von *Schiff und Mendlowicz*, *Schiff und Hübener*. Vergleiche auch mit der Arbeit von *Welcker* und der von *Fischer*.

Schliesslich sei erwähnt, dass man der Frage der Konstanz der Emp-

*) Die Tabellen V und VI wurden wegen Mangel an Zeit nicht in meinem Vortrage auf dem Nordischen Pathologenkongress in Uppsala im Juli 1947 vorgeführt, wohl aber in der Sitzung der Societas pro Flora et Fauna fennica im Mai 1947. Wenn die Titergrenze der Paarlinge dieselbe war, wurde die Differenz mit 0 bezeichnet. Wenn die Titergrenze dieselbe war, aber bei dem einem Paarlinge mit +, bei dem anderen mit ± bezeichnet war, wurde die Differenz mit 1/2 bezeichnet. Wenn die Differenz eine ganze Verdünnungsgrad entsprach wurde die Differenz mit 1 Stufe bezeichnet usw.

Aus Tabelle IV ist zu ersehen, dass die Verhältniszahlen in Finnland und Dänemark verschieden sind. Die Mittelwerte für Dänemark sind von mir aus den Zahlen von *Thomsen* u. *Kettels* ausgerechnet. Aus Zahlen von *Schiff* kann weiter ausgerechnet werden, dass die Verhältniszahl $\frac{O \text{ Anti-A}}{O \text{ Anti-B}}$ in Berlin eine andere als in Finnland und

eine andere als in Dänemark ist. Eine kleine Serie von unseren Assistenten, Dr. *T. Savolainen*, behandelt die Unterschiede zwischen Finnen und Deutschen in dieser Hinsicht. Sein interessantes Material ist jedoch auch ganz klein. Wir haben in Finnland Leute mit schwedischen und finnischen Namen betreffs der Serumstärke untereinander verglichen, ohne nennenswerte Unterschiede zu finden. Nur betreffs O Anti-A₂ war ein kleiner Unterschied vorhanden.

In der Tabelle IV habe ich auch die Frequenzzahlen der Qualitäten und deren Verhältnisse unter sich angegeben. Also die Anzahl z. B. der A-Individuen im Verhältnis zur Anzahl der B-Personen usw. Die aus der Tabelle ersichtliche Korrelation zwischen den Verhältniszahlen der Titerwerte und den Verhältnissen der Frequenz möchte ich als scheinbare und zufällige Korrelationen betrachten, weil dieselben bis auf weiteres unerklärlich und unverständlich zu sein scheinen.

In diesem Zusammenhange möchte ich noch die Frage der Vererbung der verschiedenen Quantitäten in aller Kürze behandeln.

Diesbezügliche Fragen können auf verschiedenen Wegen behandelt werden. Die Zwillingsforschung ist ein Weg, die Familienforschung ein anderer. Die Zwillingsforschung haben *Dahr*, *Bühler* und andere benutzt. Den Weg der Familienforschung hat u. a. bei uns *K. O. Renkonen* betreten.

Dahr hat nachgewiesen, dass die Variationen der Titerstärke bei eineiigen Zwillingen deutlich kleiner sind als bei den zweieiigen. Dasselbe hat er auch betreffs der Empfindlichkeit der Blutkörperchen bei E. Z. und Z. Z. gefunden, obwohl hier die Unterschiede nicht so deutlich hervortreten wie betreffs der Titerstärke des Serums. *Dahr* hat nicht angegeben, wie stark die Titerwerte und wie empfindlich die Blutkörperchen bei den untersuchten Zwillingen in seinen Serien waren. Dieses wäre doch wichtig zu wissen, um die Beweiskraft seiner Versuche beurteilen zu können. Aus unserem Material I, welches kein Zwillingsmaterial ist und nicht einmal befreundete Leute umfasst, habe ich die Chancen der Ähnlichkeit und Verschiedenheit der Titer zweier in der Reihe nacheinander folgender Personen in unseren Serien zusammengestellt, Tabelle VIII u. IX. Die Chancen der Ähnlichkeit oder Verschiedenheit sind von der Titerstärke abhängig. Die Unterschiede der Titerstärke der Paare wurden auch hier wie bei *Dahr* in Stufen berechnet.

Tabelle VIII.

Die Differenzen in Stufen in Prozent angegeben.

	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	$4\frac{1}{2}$	5	
O Anti-A	23	16	27	9	18	4	3					= 100
	66 % ₀			34 % ₀								
B Anti-A	26	13	26	11	13	3	5	2	—	—	1	= 100
	65 % ₀							35 % ₀				
O Anti-B	28	10	39	6	13	2	2					= 100
	77 % ₀			23 % ₀								
A Anti-B	30	11	31	6	13	5	4					= 100
	72 % ₀			28 % ₀								

Aus Tabelle VIII ist ersichtlich, dass die Differenzen zwischen zwei nach einander wahllos in der Reihe folgenden Individuen ziemlich gross sind. Wenn man annimmt, dass eine Verschiedenheit von einer Stufe die Grösse der technischen Versuchsfehler angibt, haben wir doch eine Ähnlichkeit von 66 % für O anti A und 77 % für O anti B gefunden.

Die in der Tabelle IX referierten Paarlinge waren so gegeneinander gestellt, dass zuerst aus den Protokollen Personen mit der Titerstärke $\frac{1}{2}$ herausgenommen wurden, und als ihre Paarlinge wurden wahllos die folgenden in der Reihe — unabhängig davon, welche Titerstärke sie hatten — genommen und deren Differenzen berechnet. In derselben Art wurden diejenigen mit dem Titer $\frac{1}{4}$ herausgenommen und mit ihren Nachfolgern verglichen usw.

Tabelle IX.

Die Differenzen in Stufen.

	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	$4\frac{1}{2}$	5	$5\frac{1}{2}$	6	$6\frac{1}{2}$	7	
$\frac{1}{2}$	—	3	3	1	8	12	25	8	10	6	11	6	2	1	4	= 100
	6 % ₀								94 % ₀							
$\frac{1}{4}$	2	—	12	1	49	1	16	3	13	2	1	—	—	—	—	= 100
	14 % ₀								86 % ₀							
$\frac{1}{8}$	11	2	52	1	14	2	15	2	1	—	—	—	—	—	—	= 100
	65 % ₀								35 % ₀							
$\frac{1}{16}$	43	11	32	1	13	—	—	—	—	—	—	—	—	—	—	= 100
	86 % ₀								14 % ₀							
$\frac{1}{32}$	20	8	52	4	12	3	1	—	—	—	—	—	—	—	—	= 100
	80 % ₀								20 % ₀							
$\frac{1}{64}$	9	8	12	5	33	7	20	3	3	—	—	—	—	—	—	= 100
	29 % ₀								71 % ₀							
$\frac{1}{128}$	—	4	10	5	18	1	39	1	13	1	2	—	—	—	—	= 100
	20 % ₀								80 % ₀							

Die Tabelle IX zeigt, dass wenn man die Individuen mit einer Titerstärke 1 : 2 mit ihren Nachfolgen in der Reihe vergleicht, man nur in 6 % eine ähnliche Reaktionsstärke erreicht. Wenn von Individuen mit einer Reaktionsstärke 1 : 16 jedes mit seinen Nachfolgern verglichen wird, findet man in 86 % eine Übereinstimmung. Die Übereinstimmung wird wieder schlecht, wenn Leute mit starken Sera 1 : 128 mit ihren Nachfolgern verglichen werden. Das Ähnlichkeitsprozent sinkt nämlich wieder. Die Zahlen der Ähnlichkeit bei den eineiigen Zwillingen sind zwar grösser bei *Dahr* als die Ähnlichkeit in unserer Tabelle VIII. Die Serien von *Dahr* sprechen somit für eine gewisse Bedeutung der Heredität, heweisen aber nicht, dass eine solche vorliegt. Es ist weiter die Diagnose der erwachsenen E. Z. oder Z. Z. immer etwas unsicher und basiert sozusagen wenigstens teilweise auf einem Circulum in der Definition.

Die Vererbung der Titerstärke der Sera und der Empfindlichkeit der Blutkörperchen kann mit Hilfe der Familienforschung in folgender Art beleuchtet werden.

Wie bekannt, können die Genotypen der einzelnen Mitglieder einer Familie in vielen Fällen festgestellt werden, wenn die Phänotypen aller Mitglieder der Familie zuerst bestimmt worden sind. Wenn z. B. der eine der Eltern ein A₁-Mensch ist und der andere ein O-Mensch, und wenn unter den Kindern sowohl A- als O-Kinder vorkommen, so kann geschlossen werden, dass der A-Vater oder die A-Mutter genotypisch ein AO-Individuum ist und dass dann die eventuellen A-Kinder genotypisch AO-Kinder sind. Die A-Eigenschaft der Kinder kann in solchen Familien nur von demjenigen der Eltern stammen, welcher ein A-Mensch ist. Die Empfindlichkeit der A-Blutkörperchen muss, wenn Vererbung der Empfindlichkeit der Blutkörperchen vorliegt und das Kind genügend alt ist, bei Kind und Eltern identisch sein. Dasselbe gilt mutatis mutandis betreffs der B-Eigenschaft in Familien, wo der Vater oder die Mutter B-Menschen sind und die Kinder von sowohl B- als O-Typus sind usw. Wenn einmal die Eigenschaft der Blutkörperchen bei den Kindern und bei Vater oder Mutter identisch sein muss, würde man erwarten, dieses beim Titrieren mit demselben Anti-Sera hervortreten zu sehen.

Ich habe teilweise zusammen mit *K. Penttinen*¹⁾ 21 Familien in der Art untersucht, dass die Empfindlichkeit der Blutkörperchen aller A- und B-Mitglieder derselben Familie mit Sera aus derselben Familie und mit fremden Sera gleichzeitig am selben Tage ausgetitriert und als Paaringe einander gegenübergestellt wurden (Material III). Die Differenzen wurden in Stufen berechnet. (Tabelle XI).

1) Die Arbeit wird fortgesetzt und in *Annal. med. experim. et biol. Fenniae* in nächsten Zukunft erscheinen.

Tabelle X.

Die vorkommenden Genotypen der Familienmitglieder (Bernstein).

	Vater	Mutter	Vorgekommene Kinder	A	B
I	BB.s BO	AO	BO BO BO	—	—
II	AO	BO	BO OO AO AB	id. ²⁾	versch.
III	AAs. AO	BO	AO AB AO	versch. ³⁾	»
IV	AO	BBs. BO	AB BO BO BO	id.	versch.
V	AAs. AO	BO	AO	versch.	id.
VI	OO	AO	AO OO AO OO	id.	—
VII	AO	AO	OO OO AAs. AO	versch.	—
VIII	AO	AO	OO OO	—	—
IX	AAs. AO	OO	AO AO	versch.	—
X	AO	OO	OO AO OO	id.	—
XI	AAs. AO	AAs. AO	AAs. AO, AAs. AO, AAs. AO	versch.	—
XII	OO	BO	OO OO OO BO	—	id.
XIII	BO	BO	OO, BBs. BO, OO, BBs. BO, OO	—	versch.
XIV	AB	OO	BO BO AO	id.	id.
XV	OO	AO	AO OO	«	—
XVI	OO	AO	AO OO AO AO OO	«	—
XVII	AO	BO	BO AB AO BO AB	»	id.
XVIII	AO	AO	OO, AAs. AO, AAs. AO, OO, AAs. AO	versch.	—
XIX	AO	AO	AAs. AO, AAs. AO, AAs. AO, OO	«	—
XX	AO	OO	OO OO AO AO OO	id.	—
XXI	OO	AAs. AO	AO AO AO	versch.	—

Aus der Tabelle X geht hervor, in welchen Familien die A- resp. B-Eigenschaft der Blutkörperchen bei den Kindern und Eltern identisch sein muss und in welchen sie identisch oder verschieden sein kann.

In der Tabelle XI sind die A-Blutkörperchen sowohl die der Eltern und Kinder als die der Kinder unter sich als Paarlinge gegenüber einander gestellt. In der Gruppe I sind die Paarlinge genetisch betrachtet identisch, in der Gruppe II können sie identisch oder verschieden sein (Siehe die Tabelle X).

Aus der Gruppe II sind die Familien XI, XVIII und XXI in welchen sowohl die A₁- als die A₂-Eigenschaft bei den Eltern vorkommen und somit auch bei den Kindern vorhanden sein können, weggelassen. In dieser Art ist Gruppe III entstanden.

In der Gruppe IV sind die A-Blutkörperchen von nicht verwandten Personen als Paarlinge gegenüber einander gestellt; also Vater und Mutter, Vater oder Mutter mit ganz fremden Personen und solche unter sich.

Die Zahlen in der Gruppe V betreffen dieselben Blutkörperchen wie in Gruppe IV. In Gruppe V₁ diejenigen Paarlinge welche sicher oder wahrscheinlich verschieden also A₁ und A₂-Individuen sind und die in der Gruppe IV eingehen, zusammengeführt. Die Gruppe V₂ umfasst die Paarlinge, welche sicher oder wahrscheinlich A₁—A₁,

¹⁾ S = seu = oder.

²⁾ id. = Die Blutkörperchen müssen identisch sein.

³⁾ versch. = Die Blutkörperchen können verschieden sein.

oder A_2-A_2 sind. Die Gruppe V_3 enthält den Rest also sowohl A_1-A_2 als A_1-A_1 und A_2-A_2 Paare. Die Untergruppen der Gruppe V sind somit qualitativ einheitlicher als die ganze Gruppe IV. Leider wurde die Differenzierung A_1 oder A_2 in diesem Materiale nur teilweise durchgeführt, weshalb auch die Untergruppen V_1 und V_2 vielleicht einige nicht richtig diagnostizierte A_1 - und A_2 -Paarlinge enthalten kann.

Die Empfindlichkeitsdifferenz jeder zwei mit einander als Paar verglichenen Blutkörperchen wurden in allen fünf Gruppen aus dem mit demselben Serum erhaltenen Resultat festgestellt. Jedes Paar wurde aber mit mehreren fremden oder verwandten Sera untersucht und somit wurde für den einzelnen Paaren mehrere Differenzen in Stufen berechnet. Die bei den Ablesungen mit + oder \pm vermerkten Endtitrationswerte wurden wie früher (Tabelle V) nicht als gleichwertig betrachtet. Die Differenzen in Stufen wurden somit nicht mit ganzen Zahlen 0, 1, 2, 3 usw. sondern mit 0, $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2 usw. berechnet.

Tabelle XL.

Die Empfindlichkeit der A-Blutkörperchen.

Gruppe I.

Eltern: AO--OO oder AB--OO oder AO-BO, Alle A-Kinder AO.

Die A-Kinderpaare müssen betreffs A unter sich identisch sein, ebenso die A-Kinder-Elternpaare.

Differenzen in Stufen.

	0	1½	1	1½	2	2½	3	3½	4	4½	5	5½
II	2	1	—	—	—	—	—	—	—	—	—	—
VI	2	2	—	1	—	—	—	—	—	—	—	—
X	5	4	—	—	—	—	—	—	—	—	—	—
XV	3	4	1	—	—	—	—	—	—	—	—	—
XVI	13	15	20	5	1	—	—	—	—	—	—	—
XVII	3	1	—	—	—	—	—	—	—	—	—	—
XX	11	14	8	—	—	—	—	—	—	—	—	—
	39	41	31	6	1	—	—	—	—	—	—	—
	111 = 91.1 %					7 = 5.9 %						

Gruppe II.

Eltern: AO \times AO, AA \times AO, AA \times OO, AA \times BO. Die A-Kinder AA oder AO.

Die A-Kinderpaare unter sich und ebenso die Kinder-Elternpaare können identisch oder verschieden sein.

Differenzen in Stufen.

	0	1½	1	1½	2	2½	3	3½	4	4½	5	5½
III	2	8	2	—	—	—	—	—	—	—	—	—
V	1	1	1	—	1	—	—	—	—	—	—	—
VII	8	3	2	—	1	—	—	—	—	—	—	—
IX	3	9	8	1	—	—	—	—	—	—	—	—
XI	11	17	7	3	5	3	3	1	2	1	1	—
XVIII	16	19	19	6	7	7	5	2	—	—	—	—
XIX	20	30	10	1	2	—	—	—	—	—	—	—
XXI	10	8	6	4	3	6	2	2	1	—	—	—
	71	95	55	15	19	16	10	5	3	1	1	—
	221 = 75.9 %						70 = 24.1 %					

Gruppe III.

Dieselbe wie Gruppe II. Die Zahlen der Familien XI, XVIII und XXI, wo A₁—A₂-Paare vorkommen, sind eliminiert.

Differenzen in Stufen.

0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	5 1/2
34	51	23	2	4							
104 = 94.7 %						6 = 5.3 %					

Gruppe IV.

Nicht verwandte A*)-Paare: Vater-Mutter, Eltern-Fremde, Fremde-Fremde.

Differenzen in Stufen.

0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	5 1/2
32	45	58	31	36	11	25	15	9	6	4	3
135 = 48.0 %						146 = 52.0 %					

*) A kann A, oder A₂ sein.

Gruppe V.

Dieselbe wie IV in Untergruppen: V₁, V₂ und V₃ geteilt.

Differenzen in Stufen.

0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4	4 1/2	5	5 1/2
Gruppe V ₁ = A ₁ mit A ₂ verglichen.											
3	2	4	10	18	11	17	14	9	6	4	3
9 = 8.9 %						92 = 90.1 %					

Gruppe V₂ = A₁ mit A₁ oder A₂ mit A₂ verglichen.

19	31	9	6	4	4	1	—	—	—	—	—
78 = 83.9 %						15 = 16.1 %					

Gruppe V₃ = A*) mit A*) verglichen.

10	12	26	15	14	2	7	1	—	—	—	—
48 = 55.2 %						39 = 44.8 %					

*) A kann A, oder A₂ sein.

Aus der Tabelle XI geht hervor, dass, wo die Empfindlichkeit der A-Blutkörperchen (A₁ oder A₂) identisch sein muss, man auch ziemlich identische Resultate erhält, nämlich die gleiche Empfindlichkeit in 94,1 %, wenn die Grösse der technischen Versuchsfehler einen Unterschied von einer Stufe nicht übersteigt. Man muss aber vielleicht mit Versuchsfehlern von der Grösse 1 1/2 Stufe rechnen. In diesem Falle wäre die Identität zwischen Kindern und Eltern über 99 %. Andererseits hat man in der Gruppe II, wo theoretisch ein quantitativer Unterschied zwischen Kindern und Eltern vorhanden sein kann (A₁ und A₂), eine bedeutend kleinere Übereinstimmung, nur in 75,8 %. Wenn die drei Familien, nämlich XI, wo die Eltern sicher verschieden (A₁ und A₂) sind, Familie XVIII, wo dasselbe wahrscheinlich der Fall ist und Familie XXI, wo die Mutter offenbar ein A₁A₂ Mensch ist, weglassen werden, so ist die Übereinstimmung auch in dieser Gruppe

gross, 94,7-prozentig. Wenn man weiter auch hier technische Fehler von $1\frac{1}{2}$ Stufen annimmt, wird das Prozent der Übereinstimmung über 96 %. Mit denselben Sera gemessen ist die Empfindlichkeit der Blutkörperchen innerhalb derselben Typengruppe, also bei A_1 - und A_1 -Paarlinge, ebenso bei A_2 - und A_2 -Paarlinge bei den in genetischer Hinsicht qualitativ identischen Personen mit einander ähnlich in 94,1 %, bis 99 % Gruppe I. In solchen Fällen, wo das Blut einer A_1 -Person als Paarling gegenüber eine A_2 -Person gestellt wird, sind grosse Unterschiede vorhanden. Vielleicht könnte man, wenn grosse Unterschiede in A-Familien gefunden werden, die Diagnose A_1 oder A_2 auf diesem Wege stellen. Doch muss man bedenken, dass in diesem kleinen Familien Material zwischen den A_1 -Eltern keine grössere Unterschiede betreffs, der Empfindlichkeit der Blutkörperchen vorgekommen war. Mit grösseren A_1 -Material wären grössere Unterschiede zwischen den Eltern und dem gemäss auch bei der Kindern zu erwarten. Dafür spricht die Familie XIX wo ein Differenz von 1 Stufe bei den Eltern vorhanden war.

Es scheint als wären die verschiedenen Werte, die man beim Auslitrieren der B- oder O-Sera mit A-Blutkörperchen derselben Gruppen A_1 oder A_2 in derselben Familie erhält, hauptsächlich auf Verschiedenheiten der Sera zurückzuführen. Wenn in einer Familie die Blutkörperchen von A_1 -Typus sind, scheint man unabhängig davon, welchen A_1 -Blutkörperchen man als Massstab benutzt, ungefähr dieselben Werte der Titerstärke eines Serums zu erhalten. Werden A_2 -Blutkörperchen als Indikator benutzt, erhält man andere Werte, aber auch hier scheint die Hauptursache der Verschiedenheiten im Serum zu liegen. Wenigstens ist es uns nicht mit unserer Technik gelungen, sichere quantitative Unterschiede betreffs der Empfindlichkeit der Blutkörperchen bei den zur selben Familie hörenden A-Paarlinge welche qualitativ derselben Art A_1 und A_1 oder A_2 und A_2 sind, zu finden. Mit genauerer Technik wird es vielleicht möglich. Bei nicht verwandten A_1 — A_1 und A_2 — A_2 Paare (Tabelle XI Gruppe V_2) ist die Übereinstimmung nun 83,9 %. Bei den gleichzeitig untersuchten Familien Mitglieder war die entsprechende Prozent 94 bis 95 %.

Somit könnte man innerhalb den Typengruppen von einer Vererbung der Empfindlichkeit der A-Blutkörperchen sprechen. Doch sei bemerkt, dass die Diagnose A_1 oder A_2 in der Gruppe V_2 teilweise nur eine Wahrscheinlichkeitsdiagnose war. Die Zahlen in dieser Gruppe entsprechen diejenigen Zahlen, 85 %, welche *Thomsen*, dort wo A_1 und A_2 Blutkörperchen offenbar gemischt untersucht waren gefunden hat. Vielleicht wird man mit Hilfe diesartigen in grösserem Umfange ausgeführten Untersuchungen eine endgültige Antwort auf die Frage erreichen. In der Tabelle XII sind die B-Blutkörperchen sowohl die der Eltern und Kinder als die der Kinder unter sich als Paarlinge gegenüber einander gestellt.

Tabelle XII.

Die Empfindlichkeit der B-Blutkörperchen.

Gruppe I.

Eltern BO—OO oder BO—AO
oder AB—OO.

Alle B-Kinder = BO.

Die B-Eltern Kinderpaare unter die B-
Kinderpaare müssen identisch sein.
Differenzen in Stufen.

Familie	0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4
I	—	—	—	—	—	—	—	—	—
IV	—	—	—	—	—	—	—	—	—
XIII	—	—	—	—	—	—	—	—	—
XIV	4	2	1	—	—	—	—	—	—
XVII	7	8	4	2	—	—	—	—	—
	11	10	5	2					
	26 = 92.1 %			2 = 7.9 %					

Gruppe II.

Eltern BB—AO, BO—BO, BB—OO.

Die B-Kinder BB oder BO.

Die B-Eltern Kinderpaare und die B-
Kinderpaare können identisch oder
verschieden sein.

Differenzen in Stufen.

Familie	0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4
I	5	9	8	2	—	—	—	—	—
IV	—	1	3	—	—	—	—	—	—
XIII	8	—	7	—	—	—	—	—	—
XIV	—	—	—	—	—	—	—	—	—
XVII	—	—	—	—	—	—	—	—	—
	13	10	18	2	—	—	—	—	—
	41 = 95.3 %			2 = 4.7 %					

Gruppe III.

Nicht verwandte Paare: Vater—Mutter Paare,
Eltern—Fremde Paare, Fremde—Fremde Paare.

Differenzen in Stufen.

0	1/2	1	1 1/2	2	2 1/2	3	3 1/2	4
51	44	19	3	1	—	—	—	—
114 = 96.6 %				4 = 3.4 %				

In der Gruppe I (Tabelle XII) sind die Paarlinge betreffs den Blutkörperchen genetisch identisch, in der Gruppe II können sie identisch oder verschieden sein (Tabelle X). Wahrscheinlich sind sie identisch.

In der Gruppe III sind B-Blutkörperchen von nicht verwandten Personen gegenüber einander als Paare gestellt. Sonst ist die Tabelle XII mit Tabelle XI vergleichbar.

Aus der Tabelle XII ist betreffs der B-Blutkörperchen eine noch grössere Ähnlichkeit der Empfindlichkeit als aus der Tabelle XI betreffs der A-Blutkörperchen ersichtlich. Wenn man technische Fehler von einer ganzen Stufengrösse annimmt, ist die Übereinstimmung in unserer Familienmaterial 92,1 bis 95,3 % und bei Nichtverwandten 96,6 %. Wenn diesartige Fehler 1 1/2 Stufe entsprechen können, was bei unserer Technik möglich ist, ist Übereinstimmung in 100 % vorhanden bei den Verwandten und bei den Nichtverwandten in 99 %.

Wenn man die ev. Fehlergrenze kleiner als 1 Stufe setzt, findet man bei den genetisch identischen eine Übereinstimmung in 75 % und nur in 52,2 bei den genetisch verschiedenen. Also eine zweifelhafte Andeutung zur Heredität.

Unser Familienmaterial ist klein. Die Blutkörperchen jedes Eltern-Kindpaares wurden mit verschiedenen starken Sera untersucht und deshalb ist die Anzahl der Paare in den Tabellen XI und XII verhältnismässig gross, während die tatsächliche Anzahl der verschiedenen

Blutkörperchenpaare bedeutend kleiner ist, wie aus der Tabelle X hervorgeht. Das kleine Material berechtigt somit zu keinen Schlüssen betreffs der Erbllichkeit der Empfindlichkeit der B-Blutkörperchen.

Weil unsere Untersuchungen in dieselbe Richtung gehen, scheint es wahrscheinlich, dass die eben besprochenen Resultate einen Fingerzeig in die Richtung bedeuten, dass eine Erbllichkeit der Empfindlichkeit der A-Blutkörperchen zwar vorliegt. Diese scheint aber qualitativer, und nicht quantitativer Art zu sein, also A_1 oder A_2 , aber nicht »stark empfindliches« A_1 oder »schwach empfindliches« A_1 und nicht »stark empfindliches« A_2 oder »schwach empfindliches« A_2 . Eine Erbllichkeit der Empfindlichkeit der B-Blutkörperchen ist schon deshalb mit unserer Technik nicht nachweisbar gewesen, weil wir überhaupt bei diesen Blutkörperchen keine nennenswerten Unterschiede auch bei den Nich-verwandte Paarlinge desselben Tages gefunden haben.

Unsere Titrierungen betreffs der Empfindlichkeit der AB-Blutkörperchen haben nicht so eindeutige Resultate gegeben, Tabelle XIII. Wird auch hier eine Differenz von 1 Stufe als möglicherweise fehlerhaft bestimmt angenommen, findet man eine Übereinstimmung in 90 % betreffs A. Wenn eine Differenz von $1\frac{1}{2}$ Stufe noch als möglicherweise fehlerhaft betrachtet werden muss, ist eine Ähnlichkeit un-

Tabelle XIII.

Die Empfindlichkeit der AB-Blutkörperchen.

Die AB-Eltern mit den A- und B-Kindern, die AB-Kinder mit den A- oder B-Eltern und die AB-Kinder unter sich verglichen.

Gruppe I.

Die Paare müssen betreffs A identisch sein.

Differenzen in Stufen.

Familie	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
II	1	5	—	—	—	—	—
III	—	—	—	—	—	—	—
IV	1	—	2	—	1	—	—
XIV	—	1	2	1	1	1	—
XVII	7	12	5	—	—	—	—
	9	18	9	2	1	1	—
	36 = 90.0 %			4 = 10.0 %			

Gruppe II.

Die Paare können betreffs A identisch oder verschieden sein.

Differenzen in Stufen.

Familie	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
II	—	—	—	—	—	—	—
III	1	1	2	3	1	1	—
IV	—	—	—	—	—	—	—
XIV	—	—	—	—	—	—	—
XVII	—	—	—	—	—	—	—
	1	1	2	3	1	1	—

Die Paare müssen betreffs B identisch sein.

Differenzen in Stufen.

Familie	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
II	—	1	—	—	—	—	—
III	—	2	3	—	—	—	—
IV	—	—	—	—	—	—	—
XIV	—	3	3	2	1	1	—
XVII	7	14	13	5	—	3	—
	7	23	19	7	1	4	—
	49 = 80.3 %			12 = 19.7 %			

Die Paare können betreffs B identisch oder verschieden sein.

Differenzen in Stufen.

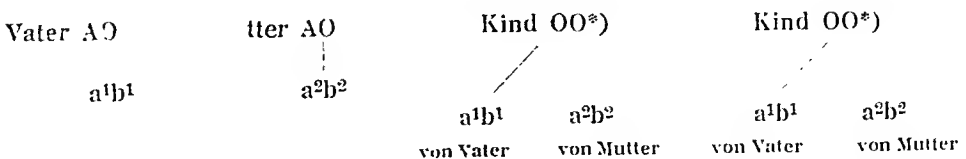
Familie	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
II	—	—	—	—	—	—	—
III	—	—	—	—	—	—	—
IV	1	1	2	1	—	1	—
XIV	—	—	—	—	—	—	—
XVII	—	—	—	—	—	—	—
	1	1	2	1	—	1	—

ter den identischen A-Werten der AB-Blutkörperchen der Kinder und Eltern in 95,0 % vorhanden. Die entsprechenden Prozente für die B-Werte in AB-Familien sind 80,3 resp. 91,8 %. Bei den Personen, wo die A- und B-Anteile der AB-Blutkörperchen verschieden sein können, sind die Unterschiede vielfach grösser. Betreffs der Vererbung der A- und B-Anteile in AB-Blutkörperchen sind keine Schlüsse möglich. Die Anzahl der untersuchten Blutkörperchen ist klein und Abweichungen sowohl betreffs des A- als B-Anteils kommt auch bei den jüngeren »identischen« Paaren vor (Tabelle XIII).

Ich habe schliesslich auch einige Versuche betreffs der Heredität der agglutinierenden Stärke der Sera ausgeführt. Ganz wie die Heredität der Empfindlichkeit der Blutkörperchen mit Hilfe Familienmaterials beleuchtet werden kann, kann es auch die Vererbung der Titerstärke der Sera. Wenn z. B. beide Eltern A_1 -Menschen sind und O-Kinder in dieser Familie vorkommen, sind beide Eltern genotypisch A_1O -Menschen. Eventuell vorkommende O-Kinder haben die O-Eigenschaft von Vater und Mutter bekommen und können unter sich betreffs der Heredität der agglutinierenden Stärke der anti-A und anti-B verglichen werden.

Es existieren in der Hauptsache zwei Theorien für die Vererbung der Agglutininene. Die eine Theorie ist von *Bernstein* aufgestellt, die andere von *Furuhata*. Vergleiche *Wiener*. Wenn die Theorie von *Furuhata*, dass A mit b, B mit a und a mit b völlig gekoppelt sind (Ab, aB und ab) (complete linkage) richtig wäre, müsste m. E. in Familien, wo beide Eltern AO-Individuen oder beide BO-Individuen sind, eventuell vorkommende O-Kinder, welche immer Homozygoten (ab). (ab) sein müssen und nie die Genpaare Ab oder Ba erben, betreffs sowohl Anti A = α als Anti B = β identisch sein, wie das beistehende Schema (*Furuhata*) es zeigt.

Schema 2. (Furuhata).



Dasselbe Schema ist für Eltern BO und BO betreffs der ab-Gene bei den OO Kindern gültig.

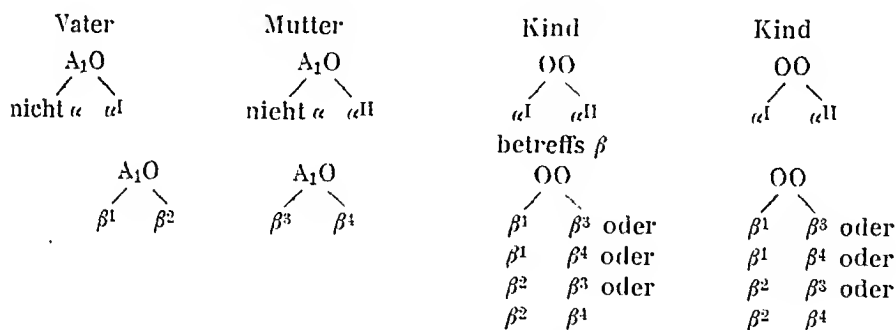
Die erste von mir untersuchte Familie dieser Art zeigte, dass α zwar bei beiden O-Kindern identisch war, β aber nicht. Meine späteren Versuche haben dieses Resultat bestätigt. Entweder existiert ab nicht wie *Furuhata* annimmt, als völlig gekoppeltes Genpaar, oder sind die a-

* Die O-Kinder müssen genetisch identisch sein, wenn α mit β gekoppelt vererbt werden.

und b-Eigenschaften nur qualitativ aber nicht quantitativ gekoppelt vererbbar.

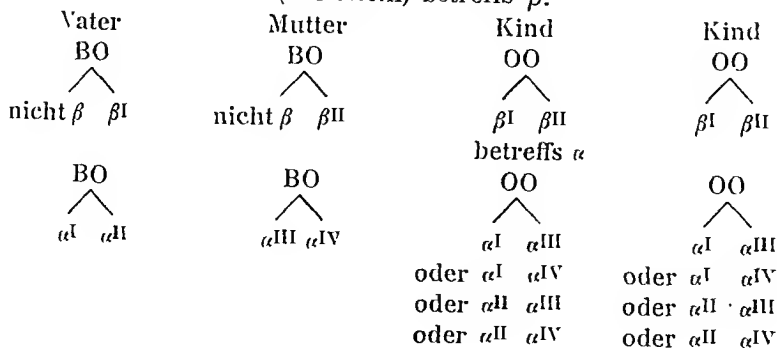
Bernstein hat angenommen, dass sowohl α als β voneinander unabhängige normale Bestandteile der menschlichen Sera sind. Wo die Blutkörperchen von A_1 -Natur sind, kann α nicht im Serum entwickelt werden, wohl aber β . Sind die Blutkörperchen von B-Natur, kann β nicht entwickelt werden wohl aber α . Demgemäss müssen, wo beide Eltern A_1O sind, die O-Kinder betreffs α identisch sein. Die β -Eigenschaft könnte variieren (Schema 3). Wo beide Eltern BO sind, müsste vice versa die β -Eigenschaft bei den O-Kindern identisch sein und könnte die α -Eigenschaft variieren (Schema 4). Umgekehrt könnte man sagen, dass wenn die O-Kinder in den AO- und AO-Familien betreffs der Stärke des α -Agglutinins und in BO-BO-Familien betreffs β immer quantitativ identisch sind, würde dieses für die Vererbung der Quantitäten sprechen. Meines Erachtens ist die von Bernstein aufgestellt Theorie am besten so zu verstehen dass auch die α - und β -Eigenschaften von bestimmten Genpaare $\alpha\alpha$ und $\beta\beta$ reguliert werden. Diese Genpaare sind nicht mit den die Bluttypen A, B und O regulierende Gene gekoppelt. Die Konstanz in den Tabellen I und XIV ist nur so verständlich.

Schema 3. (Bernstein) betreffs α .



Somit muss α bei den O-Kindern identisch, β kann verschieden sein. Auf die Frage α_1 und α_2 scheint es mir nicht notwendig in diesem Zusammenhange näher einzugehen. Siehe doch das Schema 5.

Schema 4. (Bernstein) betreffs β .



Betreffs β ist die Sache bei O-Kinder in BO-BO-Familien noch klarer als in den AO-AO-Familien betreffs α , weil man nicht in der B-Gruppe mit zwei Untergruppen (B_1 und B_2), wie in der A-Gruppe (A_1 und A_2) zu tun hat. In BO-BO-Familien ist bei den O-Kindern β identisch, α kann verschieden sein, Schema 4.

Ich habe unter im ganzen 76 austitrierten Familien nur vereinzelte dieser Art zur Verfügung gehabt. Die Sera der 12 O-Kinder wurden mit A_1 und A_2 und B-Blut verschiedener Provenienz austitriert und nur die mit denselben Blutkörperchen am selben Tage in der selben Familie erhaltenen Titrierungsergebnisse miteinander als Paare verglichen. Siehe die Tabellen XIV.

Tabelle XIV. 6 Kinder.
O-Kinderpaare; Eltern AO und AO oder BO und BO.

Die Stärke der Sera mit theoretisch identischen Agglutininen.										Die Stärke derselben Sera mit verschiedenen oder identischen Agglutininen.									
α -Agglutinine. Differenzen in Stufen.										β -Agglutinine. Differenzen in Stufen.									
Familie	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$		0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$		
VII	3	—	3	—	—	—	—	—		—	—	—	—	1	1	1	—		
VIII	2	2	2	—	—	—	—	—		—	—	—	—	1	2	1	—		
XLII	1	1	—	—	—	—	—	—		1	1	—	—	—	—	—	—		
	6	3	5							1	1	—	—	2	3	2	—		
	14									2					7				
β -Agglutinine.										α -Agglutinine.									
XIII	3	—	3	—	—	—	—	—		—	1	—	—	1	—	—	1		
Zusammen.										Zusammen.									
	9	3	8	—	—	—	—	—		1	2	—	—	3	3	2	1		
	100 %									3 = 72.4 %				8 = 27.6 %					
α -Agglutinine.										β -Agglutinine.									
XVIII	10	5	6	2	3	—	2	1		1	5	2	3	1	—	1	1		
	21 = 72.4 %			8 = 27.6 %						8 = 57.1 %				6 = 42.9 %					

Trotz der geringen Anzahl der Sera ist die Theorie der voneinander unabhängigen α - und β -Agglutinine (Bernstein) überall bestätigt. Wo die α - oder β -Agglutinine in der Theorie identisch sein müssen, ist dieses in fast 100 % sowohl betreffs α als β bestätigt, Tabelle XIV links. Wo die Werte der α und β bei den Paaren variieren können, ist es auch oft der Fall, Tabelle XIV rechts und auch XV. mit O-Kindern, wo die A-Eigenschaft des einen der Eltern A_2 ist. In solchen Fällen ist, die Identität der O-Kinder betreffs Anti-A nicht notwendig. Siehe das Schema 5 und die Resultate betreffs der Familie XVIII in der Tabelle XIV.

Tabelle XV.

O-Kinderpaare; Eltern AO und BO oder AO und OO oder BO und OO.
Die α - und β -Agglutinine können beiderseits theoretisch verschieden
oder identisch sein.

Familie	α -Agglutinine. Differenzen in Stufen.								β -Agglutinine. Differenzen in Stufen.							
	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$
VI	1	—	—	—	1	—	—	—	—	—	—	—	1	—	—	—
X	—	—	1	—	2	2	—	—	1	—	1	—	—	—	—	—
XII	2	4	3	—	—	—	—	—	2	—	4	—	3	—	—	—
XVI	1	1	3	—	1	—	—	—	1	—	1	—	—	—	—	—
XX	3	5	4	4	1	—	—	—	3	2	1	1	—	—	—	—
XXXIII	1	3	1	1	—	—	—	—	1	2	—	—	—	—	—	—
XXXV	2	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—
L	—	1	1	—	—	—	—	—	—	—	—	—	1	—	—	—
L1	1	1	—	—	—	—	—	—	—	1	—	—	—	—	—	—
LIII	1	2	3	—	—	—	—	—	1	—	—	—	—	2	—	—
LXIII	—	—	1	1	—	—	—	—	2	—	—	—	—	—	—	—
LXXII	—	3	1	1	1	—	—	—	—	2	—	1	—	2	1	—
LXXV1	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	—
	12	20	18	8	7	2	—	—	12	7	8	3	5	4	1	—
	50 = 74.6 %				17 = 25.4 %				27 = 67.5 %				13 = 32.5 %			

Schema 5. (Viergenentheori α_1 und α_2).betreffs α Vater
 A_2O
 α_1^I α_1^{II} oder α_2
Mutter
 A_1O
 nicht α_1 α_1^{III} oder α_2
nicht α_2
Kind
 OO
 α_1^I α_1^{III*}
oder α_1^{II} α_1^{III*}
Kind
 OO
 α_1^I α_1^{III*}
oder α_1^{II} α_1^{III*}
betreffs β A_2O β_I β_{II} A_1O β^{III} β^{IV} OO
 β^I β^{III}
oder β^I β^{IV}
oder β^{II} β^{III}
oder β^{II} β^{IV}
 OO
 β^I β^{III}
oder β^I β^{IV}
oder β^{II} β^{III}
oder β^{II} β^{IV}

Meine Versuche sprechen somit entschieden für eine Vererbung der Stärke des Agglutinine α und β . Obwohl ich unter 78 Familien nur einige verwendbare Familien gefunden habe, scheint es doch leichter auf diesem Wege ein genügend grosses für die Theorie des Isoagglutinationsphänomens verwendbares Material zu sammeln, als auf dem Wege der Zwillingsforschung, welche *Dahr* betreten hat.

Aus der Tabelle XV ist auch ersichtlich, wie die α - und β -Agglutinine bei O-Kinderpaaren in solchen Familien, wo sowohl α als β theo-

*) α^{III} kann α_1 oder α_2 sein.

retisch gleich oder verschieden stark bei den Kindern sein können aber nicht sein müssen, sich verhalten. Die Ähnlichkeit der Reaktionsstärke ist nicht so gross wie es die Tabelle XIV betreffs hereditär identischer Agglutinine zeigt. Bei den hereditär identischen α und β Agglutininen war die quantitative Stärke in 100 % dieselbe, Tabelle XIV. In Tabelle XV, wo eine hereditäre Ähnlichkeit nicht vorhanden sein müsste, war die quantitative Ähnlichkeit betreffs α nur in 74,6 % und betreffs β nur in 67,5 % zu sehen. Wo keine Heredität vorhanden war, Tabelle V, war die β -% nur 47,3.

Meine Versuche sprechen somit entschieden für eine Vererbung der Stärke der Agglutinine und somit auch für die Vererbung der serologischen Quantitäten. Die Anzahl der Versuche ist aber zu gering um endgültige Schlüsse zu erlauben. Die Bedeutung des Milieus möchte ich natürlich nicht betreffs der Isoagglutinine, auf Grund dieser vereinzelt Versuche verneinen. Bekannt ist doch dass bei erwachsenen Individuen die agglutinierende Stärke des Serums ziemlich konstant und von Krankheitszuständen und anderen äusseren Faktoren ziemlich wenig beeinflusst ist. Das Isoagglutinationsphänomen¹⁾ hat weiter in quantitativer Hinsicht auch deshalb eine Sonder- oder Schlüsselstellung, weil die beiden bei der Reaktion teilnehmenden Faktoren die Stärke des Serums und die Empfindlichkeit der Blutkörperchen jede für sich ausserhalb des Körpers in Reagenzgläser untersucht werden können, was sonst betreffs antropologisch verwendeten Zahlen selten oder nie möglich ist. Obwohl das Milieu für die Pflanzen, Tiere und Menschen in quantitativer Hinsicht sicherlich von grösster Bedeutung ist, schliesst diese Tatsache natürlich nicht jedes Vorkommen einer Vererbung der serologischen Quantitäten aus.

Man kann noch einen anderen Weg zur Beleuchtung der Vererbung der Titer versuchen. Man kann die Titerstärke der Eltern mit derjenigen der Kinder ohne Rücksicht auf die Genotypen vergleichen. Dazu muss aber auch ein ziemlich grosses Material gesammelt und der quantitative Unterschied zwischen Alten und Jungen berücksichtigt werden.

Die Reaktionsstärke der Sera nimmt ja, wie gesagt, in höheren Alter ab. Die Sera von Eltern und Kindern sind somit nicht direkt miteinander zu vergleichen. In Finnland hat *K. O. Renkonen* aus unserem Institut ein kleines Familienmaterial dieser Art veröffentlicht und dabei Zahlen erhalten, welche seines Erachtens für eine Heredität der Titer zu sprechen scheinen.

Zusammen mit Dr. *M. Tuomioja*²⁾ habe ich die agglutinierende Stärke der Sera der Eltern und Kinder von 32 Familien untersucht. In 29 Familien, wo die Eltern A- und A- oder A- and O- oder beide

¹⁾ Mit steigendem Alter sinkt zwar bei Erwachsenen die agglutinierende Stärke allmählich aber in antropologischem Alter weniger, *Thomsen*.

²⁾ Die Arbeit wird ausführlich in *Ann. med. exp. et biol. Fenniae* erscheinen.

O-Personen waren, wurde die Stärke aller β -Agglutinine ausstitriert. In 7 Familien, wo die Eltern B- und B- oder B- und O- oder beide O-Personen waren, wurde die Stärke der α -Agglutinine festgestellt. Die O-Familien waren vier und in beiden Serien dieselben. Die Titrierungen der Agglutinine wurde innerhalb den Familien hauptsächlich mit denselben Blutkörperchen ausgeführt. Wo mehrere Titrierungen mit mehreren Blutkörperchen ausgeführt wurden, wurden die Mittelwerte ausgerechnet. Die Resultate sind in der Tabelle XVI zusammengestellt.

Tabelle XVI.
A- und O-Sera mit B-Blutkörperchen ausstitriert.

	Anzahl der Familien	Mittelw. der Eltern	Anzahl der Kinder	Mittelw. der Kinder	Anzahl der Familien	Mittelw. der Eltern	Anzahl der Kinder	Mittelw. der Kinder
Mütter 1/2 — 1/23			Anti B		Mütter 1/24 — 1/256			
Väter 1/2 — 1/23	14	8.8	45	50.0	9	19.4	24	34.1
, 1/24 — 1/256	1	17.2	5	45.9	5	45.4	13	93.8
Anti A								
B- und O-Sera mit A-Blutkörperchen ausstitriert								
, 1/2 — 1/23	1	16.0	2	48.0	3	23.3	7	45.3
, 1/24 — 1/256					3	65.3	9	87.1

Aus der Tabelle XVI ist ersichtlich, dass sowohl betreffs α als β die grössten Mittelwerte bei den Kindern dort wo die Mittelwerte der Eltern die grössten waren, gefunden wurden. In einzelnen Familien und bei einzelnen Kindern wurden Abweichungen von dieser Regel notiert. Man kann somit nicht auf Grund dieser nur circa 30 Familien umfassenden und teilweise unter sich abweichenden Resultate irgendwelche endgültige Schlüsse ziehen. Doch sprechen unsere Resultate in dieselbe Richtung wie die von Dahr, K. O. Renkonen u. a. gefundenen. Die Tatsache, dass die Stärke der Agglutinationsreaktion vom Alter des Untersuchten teilweise abhängig ist erschwert doch das Beurteilen der Resultate.

Schlussfolgerungen:

Die Frage der Konstanz der isoagglutinatorischen Quantitäten ist noch nicht als gelöst zu betrachten. Doch kann man innerhalb der Typengruppen, genügend grosses Material und analoges Ablesen der Resultate vorausgesetzt, von einer relativen Konstanz betreffs der *Mittelwerte* derselben sprechen. Die Verhältniszahlen der Mittelwerte der Titer $\frac{O \text{ anti A}}{O \text{ anti B}}$ scheinen unabhängig von der Ablesungsgrenze noch mehr konstant zu sein. Material I.

Die Empfindlichkeit der Blutkörperchen innerhalb der Blutgruppen

A und B scheinen bei Erwachsenen (20—50 Jährigen) in derselben Population viel weniger variieren als die Stärke der Sera (Material II, Nichtverwandte).

Die Empfindlichkeit der B-Blutkörperchen war bei den Verwandten und auch bei Nichtverwandten Personen dieselbe in etwa 95 % in dem kleinen Familienmaterial III.

Die Empfindlichkeit der A₁- und A₂-Blutkörperchen war verschieden, aber innerhalb der Typengruppe variierten die A₁- und A₂-Blutkörperchen unter sich auch wenig. Bei Nichtverwandten Personen konnten doch Unterschiede betreffs der Empfindlichkeit der A₁- und A₂-Blutkörperchen auch innerhalb der Typengruppen nachgewiesen werden. Vorausgesetzt dass die Fehlergrenze 1½ Stufe mit unserer Technik entspricht, war eine Ähnlichkeit innerhalb der A₁-Gruppe bei den Verwandten Eltern-Kinder in etwa 95 % und bei Nichtverwandten in 83,9 % vorhanden (Material III).

Ungeachtet dieser Andeutung zur Heredität ist es nicht mit unserer Technik und kleinen Familienmaterial gelungen, eine Heredität der Empfindlichkeit der Blutkörperchen erwachsener Individuen innerhalb den Blutgruppen A₁, A₂ und B sicher festzustellen.

Mit Hilfe der Familienforschung scheint dagegen eine gewisse Heredität der agglutinierenden Stärke der Sera festgestellt werden zu können. Unser Material ist aber doch zu klein, um endgültige Schlüsse zu erlauben.

Unsere Versuche bestätigen nicht die Theorie von *Furuhata* betreffs der Isoagglutinine als genetisch gekoppelte Genpaare. Die Resultate stimmen bis jetzt aber mit der Theorie der von den Genen A und B unabhängigen α - und β -Agglutinine des normalen Serums (*Bernstein*) besser überein. Die α - und β -Agglutinine werden wahrscheinlich von zwei von einander unabhängigen Genpaare $\alpha\alpha$ und $\beta\beta$, welche auch nicht mit den die Bluttypen A, B und O regulierenden Gene gekoppelt sind, reguliert.

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EIN EIGENARTIGER, FAMILIÄRER TYPUS VON EXTREM SCHWACHEM A.-FAKTOR. »A₆«

Von Bengt Jonsson und Kristian Fast.

(Eingegangen bei der Redaktion am 12. März 1948.)

Bei im Jahre 1940 für militärische Zwecke vorgenommenen Blutgruppenbestimmungen fanden wir eine Probe von einer Person S. Sch., welche bei gewöhnlicher Routineuntersuchung der Blutkörperchen als O-Gruppe, bei der Serumkontrolle jedoch als A-Gruppe reagierte, d. h. eine starke Agglutination mit B-Blutkörperchen, aber keine Agglutination mit A₁- und A₂-Blutkörperchen gab.

In dem Glauben, dass hier eine O-Blutprobe mit α -Hämolysin das Agglutinationshemmung gab, vorlag, was die gewöhnlichste Ursache der eben angegebenen Abweichung ist, nahmen wir eine Inaktivierung des Serums vor. Das Untersuchungsergebnis war aber dasselbe. Bei Absorptionsversuchen mit den Blutkörperchen von S. Sch. konnten wir aber eine deutliche obgleich schwache spezifische Absorption des α -Agglutinins aus B-Seren nachweisen. Hier lag folglich ein Fall von ausserordentlich schwach entwickelten A-Rezeptor vor.

Aus diesem Anlasse wurden Blutproben von den nächsten Verwandten S. Sch:s (Mutter, Vater und Bruder) angefordert. Bei der Untersuchung erwies sich der Vater (Ö. Sch.) als ein gewöhnliches O-Individuum während die Mutter (L. Sch.) und der Bruder (A. Sch.) derselben »schwachen« A-Gruppe wie S. Sch. angehörten.

Die Absorptionsverhältnisse gehen aus den Tabellen I, II, und III hervor.

Die roten Blutkörperchen sowohl bei L. Sch. als bei A. Sch. und S. Sch. zeigen folglich eine ganz deutliche, wenn auch schwache, spezifische Absorption des α -Agglutinins. Wir kommen später auf die Absorptionsverhältnissen zurück. Der A-Charakter der Blutproben musste indessen als ganz sichergestellt gelten.

Tabell I.

Titer gegen A ₁ -Blkp. (B. J.) B-Mischserum (mil.) abs. mit 1/2-Vol. Blkp.					Titer gegen A ₂ -Blkp. (N-n) B-Mischserum (mil.) abs. mit 1/2-Vol. Blkp.				
Verd.	Ö. Sch.	L. Sch.	S. Sch.	O-Gruppe A. J-n.	Verd.	Ö. Sch.	L. Sch.	S. Sch.	O-Gruppe A. J-n.
1:2	+++(+)	+++(+)	+++(+)	+++(+)	1:2	+++(+)	+++(+)	+++	+++(+)
1:4	+++(+)	+++(+)	+++(+)	+++(+)	1:4	+++(+)	+++	++(+)	+++(+)
1:8	+++(+)	+++(+)	+++(+)	+++(+)	1:8	+++	++(+)	++	+++
1:16	+++(+)	+++(+)	+++	+++(+)	1:16	++(+)	+	+	++(+)
1:32	++(+)	++(+)	++(+)	++(+)	1:32	++(+)	—	—	+
1:32	++	+	++(+)	++(+)	1:64	+	—	—	—

Titer gegen A ₁ -Blkp. (B. J.) B-Mischserum (mil.) abs. mit 1/1 Vol. Blkp.					Titer gegen A ₂ -Blkp. (N-n) B-Mischserum (mil.) abs. mit 1/1 Vol. Blkp.				
Verd.	Ö. Sch.	L. Sch.	S. Sch.	O-Gruppe A. J-n.	Verd.	Ö. Sch.	L. Sch.	S. Sch.	O-Gruppe A. J-n.
1:2	+++(+)	+++(+)	+++(+)	+++(+)	1:2	+++(+)	++(+)	+++	+++(+)
1:4	+++(+)	+++(+)	+++(+)	+++(+)	1:4	+++(+)	++	++(+)	+++(+)
1:8	+++(+)	+++(+)	+++(+)	+++(+)	1:8	+++	+	+	+++
1:16	+++	+++	+++	+++(+)	1:16	++(+)	—	(+)	++(+)
1:32	+++	++(+)	++(+)	++(+)	1:32	++(+)	—	—	++(+)
1:64	++(+)	(+)	+	++	1:64	—	—	—	—

Titer gegen A ₁ -Blkp. (B. J.) B-Mischserum (mil.) abs. mit 2 Vol. Blkp.					Titer gegen A ₂ -Blkp. (N-n) B-Mischserum (mil.) abs. mit 2 Vol. Blkp.				
Verd.	Ö. Sch.	L. Sch.	S. Sch.	O-Gruppe A. J-n.	Verd.	Ö. Sch.	L. Sch.	S. Sch.	O-Gruppe A. J-n.
1:2	+++(+)	+++(+)		+++(+)	1:2	+++(+)	++		+++(+)
1:4	+++(+)	+++(+)		+++(+)	1:4	+++	+		+++(+)
1:8	+++(+)	+++		+++(+)	1:8	++(+)	—		+++
1:16	+++	++(+)		+++(+)	1:16	++	—		++(+)
1:32	++(+)	++(+)		++(+)	1:32	+	—		++(+)
1:64	++(+)	+		++	1:64	—	—		—

Bei den Agglutinationsversuchen mit den Blutkörperchen von diesen drei Personen und verschiedenen B-Seren, deren Titer gegen A₁-Blutkörperchen bis 1/256, gegen A₂-Blutkörperchen 1/64 und gegen A₂B-Blutkörperchen 1/16—1/32 betrug, und mit einem O-serum, dessen Titer nach Reinigung mit B-Blutkörperchen gegen A₁-Blutkörperchen 1/128—1/256, gegen A₂-Blutkörperchen 1/128 und gegen A₂B-Blutkörperchen 1/64 betrug, wurde gar keine Agglutination erhalten, gleichgültig ob die Versuche in Zimmertemperatur oder in der Kälte (+ 4° C) ausgeführt wurden.

Tabel III.

	Titer gegen A ₁ -Blkp, B-Serum (Mischserum) abs. mit 1/1 V. Blkp.			Titer gegen A ₂ -Blkp. B-Serum (Mischserum) abs. mit 1/1 V. Blkp.		
	O (A.J.)	A ₂ B (Sv.)	S. Sch.	O (A.J.)	A ₂ B (Sv.)	S. Sch.
1:1	+++(+)	+++(+)	+++(+)	+++(+)	+++	+++(+)
2	+++(+)	+++(+)	+++(+)	+++(+)	+(+)	+++(+)
4	+++(+)	+++(+)	+++(+)	+++(+)	(+)	+++
8	+++(+)	+++(+)	+++(+)	+++	—	++
16	+++(+)	+++(+)	+++(+)	+++	—	+
32	+++(+)	+++	+++	+(+)	—	(+)
64	+++(+)	++	+++	(+)	—	—
128	+	—	++	—	—	—
256	(+)	—	—	—	—	—

Die Blutkörperchen der drei Personen gaben dagegen mit diesen B-Seren und mit diesem O-Serum (aber nicht mit A-Seren) eine mehr oder weniger *spärliche Bildung von kurzen typischen Geldrollen* (meistens nur bis 5—6 Blutkörperchen in den Geldrollen). Diese Geldrollenbildung zeigte keine Sichere Zunahme in der Kälte (+ 4° C) und war nicht nachweisbar stärker in Seren mit höherem Titer. Schon in Serumsverdünnung 1 : 2 war die Geldrollenbildung völlig oder fast völlig aufgehoben.

Mit animalischen α_2 -Serum (anti O-haltigen Aalseren) gaben die Blutkörperchen der drei Personen starke Agglutination von demselben Stärkegrad wie Standard-O-Blutkörperchen.

Die Seren der drei Personen gaben mit B-Blutkörperchen starke Agglutinationen (Titer 1/16—1/32) aber mit A₂-Blutkörperchen gar keine Agglutination (auch nicht in der Kälte). Mit A₁-Blutkörperchen wurden wechselnde Resultate erhalten, bei einigen Untersuchungsgelegenheiten trat eine schwache Agglutination schon in Zimmertemperatur hervor, welche in der Kälte stärker wurde, bei anderen Gelegenheiten trat eine schwache Reaktion nur in der Kälte hervor und bei wieder anderen Gelegenheiten konnte gar keine Agglutination, auch nicht in der Kälte, erhalten werden. Es gab folglich bei den drei Personen ein *inkonstantes* α_1 von Kälteagglutinincharakter.

Untersuchung auf das Vorkommen von A-Substanz im Speichel der drei Personen fiel *ganz negativ* aus.

In der Literatur sind früher eine Anzahl von verschiedenen A-Faktortypen noch schwächer als A₂ beschrieben.

Die eingehendsten Untersuchungen über die schwachen A-Typen sind von Gammelgaard (1), der auch das bisher beste System für ihre Differenzierung und Benennung angegeben hat, veröffentlicht.

Wir verwenden in folgenden, wo nicht ausdrücklich anders angegeben, die Nomenklatur Gammelgaards.

Bei einem Vergleich zwischen den von Gammelgaard angegebenen Kriterien der schwachen A-Subgruppen und den von uns bei den drei A-Mitgliedern der Familie Sch. gefundenen Verhältnissen findet man keine vollständige Identität. Wir haben uns daher genötigt gesehen, den bei der Familie Sch. gefundenen A-Typus als eine besondere Subgruppe, vorläufig A_6 genannt, aufzustellen.

In Tabelle IV findet man einen Vergleich zwischen ein Blutgruppenschema Gammelgaards und den von uns mit A_6 gewonnenen Untersuchungsergebnissen.

Aus Tab. IV geht unmittelbar hervor, dass » A_6 « in vielen Beziehungen ganz wesentlich von Typus A_3 und Typus A_x abweicht. A_6

Tabelle IV.

Gammelgaards Unter-Gruppenschema				Eigene Untersuchungsergebnisse
A_3	A_4	A_5	A_x	» A_6 «
a) Ziemlich schwache Agglutin. in allen Iso-Seren. Viele nicht so kleine, aber ziemlich zarte Agglutinate und viele vollständig nichtagglutinierte Blkp. Titer etwas höher als A_2B .	Iso-Seren schwache Agglutin. mit kleinen Agglutinat, aber keine nicht agglutinierte Blkp. Titer unerheblich schwächer als A_2B .	Noch schwächere Agglutin. mit Iso-Seren als A_4 , aber derselbe Typ (also nicht A_3 Typ). Titer einige Stufen niedriger als A_4 .	Keine oder ganz geringe Agglutin. in Iso-Seren (ungefähr wie A_5B). Titer O.	Keine nachweisbare Agglutin. in Iso-Seren, nur unerhebliche Geldrollenbildung. Titer O.
b) Absorp.-Fähigkeit näher A_2 als A_2B .	Absorp. Fähigkeit etwas schwächer als A_2B .	Absorp. Fähigkeit besonders schwach, bedeutend schwächer als A_2B .	Absorp. Fähigkeit fehlt wie bei O-Blkp.	Absorp. Fähigkeit etwas schwächer als A_2B , scheint möglicherweise etwas schwächer als A_4 , aber sicher stärker als A_5 .
c) Kein α_1 im Serum.	Nicht selten α_1 im Serum	Oft α_1 im Serum, bisweilen sogar schwache Agglutin. mit A_2 -Blkp.	Kein α_1 im Serum. Keine Agglutin. mit A_2 -Blkp.	α_1 kommt vor im Serum, obwohl nicht konstant. Keine Reaktion mit A_2 -Blkp.
d) Starke Agglutin. mit anti-O.	Starke Agglutin. mit anti-O.	Starke Agglutin. mit anti-O.	Keine Agglutin. mit anti-O.	Starke Agglutin. mit anti-O.
e) A-Substanz etwas schwächer im Speichel als A_2 .	A-Substanz sehr schwach im Speichel.	A-Substanz gar nicht oder kaum nachweisbar im Speichel.	A-Substanz ebenso stark wie bei A_1 und A_2 .	A-Substanz nicht nachweisbar im Speichel.

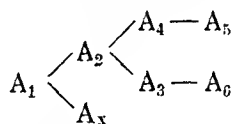
ähnelt dagegen mehr, den Typen A_4 und A_5 , obgleich auch hier erhebliche Unterschiede bestehen.

Der wichtigste Unterschied zwischen A_6 und den Typen A_4 und A_5 ist die extrem schwache Agglutinabilität, mit den zu unserer Verfügung stehenden Isoseren vielleicht richtiger das Fehlen von Agglutinabilität der A_6 -Blutkörperchen. Im interessanten Gegensatz zu dieser extrem herabgesetzten Agglutinabilität steht die Absorptionsfähigkeit, welche sich eher derjenigen A_4 :s als A_5 :s nähert.

Um die wesentliche Verschiedenheit der Agglutinabilitäten noch mehr zu beleuchten, kann folgendes angeführt werden: Eines von den Isoseren Gammelgaards zeigte einen Titer gegen A_2B von $1/32$, gegen A_4 von $1/16$ und gegen A_5 von $1/4$, während unsere Isoseren mit einem Titer gegen A_2B bis $1/64$ dagegen *gar keine Agglutination* von A_6 -Blutkörperchen gaben. Leider haben wir nicht Gelegenheit gehabt die A_6 -Blutkörperchen mit Anti-A-Immunseren oder mit einer Hämolysehemmungsmethode zu prüfen.

Von grossem theoretischen Interesse erscheint uns die schwache, aber deutliche, Tendenz der A_6 -Blutkörperchen zur Geldrollenbildung in B- und O-Seren.

Alle diese Untersuchungsergebnisse machen, wie uns scheint, die Annahme einer besonderen A_6 -Gruppe erforderlich. Das Verhältnis zwischen der verschwindenden Agglutinabilität und dem leidlich beibehaltenen Absorptionsvermögen u. a. veranlassen uns zur Aufstellung des folgenden theoretischen Schemas der Verwandtschafts- oder Entwicklungsverhältnisse innerhalb des A-Systems:



Eine direkte Identifizierung der, wie es scheint, teilweise mit Priorität angegebenen, provisorischen Gruppeneinteilung A_3 , A_4 und A_5 Hirschfelds und Amzels (2) mit den Gleichgenannten Gruppen Gammelgaards, kann nicht vorgenommen werden, aber die Benennungen dürften doch wahrscheinlich einander ungefähr entsprechen. Die von Hirschfeld und Amzel als A_4 beschriebenen Individuen haben jedoch wie es scheint vielleicht einem etwas schwächer ausgesprochenen A-Charakter als der Typus A_4 Gammelgaards.

Unter den in der Schrift Gammelgaards nicht genannten Fällen von schwachen A-Gruppen findet man einige von Wiener und Silbermann (3) und Moureau (5, 6) beschriebene Fälle wahrscheinlich A_3 , und einige Fälle von Sachs (4), wahrscheinlich A_4 .

Das Vorkommen von A_6 bei einer Mutter und ihren beiden Söhnen deutet auf eine erbliche Eigenschaft hin. Einen Schluss betreffs der Vererbungsweiss und der Dominanzverhältnisse aus dieser einzigen kleinen Geschlechtstafel zu ziehen wäre zwar verfrüht, aber aller Wahrscheinlichkeit nach liegt hier noch ein multipel alleles Gen innerhalb des A_1A_2BO -Systems vor.

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6. — Ibid. 1943, 1—2, 22.

BESTEHT KEINE INVISIBLE IMMUNISIERUNG UND PRÄZESSION BEI POLIOMYELITIS?

EIN BEITRAG ZUR FRAGE DER METHODIK BEI MORBIDITÄTSUNTERSUCHUNGEN EPIDEMISCHER KRANKHEITEN

Von *Wilh. Wernstedt*.

(Eingegangen bei der Redaktion am 12. März, 1948.)

In seiner umfangreichen Studie, »On the problem of Poliomyelitis«, deren Schwerpunkt auf die obigen Fragen verlegt ist, gelangt *Bertenius* zu der Schlussfolgerung, dass es bei der Poliomyelitis keine invisible Immunisierung und durch diese bedingte Präzession gebe. Er vertritt den Standpunkt, die bei Poliomyelitisepidemien ganz allgemein konstatierte höhere Morbidität auf dem Lande gegenüber den Städten, sowie der Anstieg der Morbidität in den jüngsten Altersklassen (Präzession) mit der Bevölkerungsdichte seien anders zu erklären als durch eine invisible Immunisierung.

Bertenius sagt (S. 155): »*Wernstedt's* studies (1917, 1939) of poliomyelitis in Sweden during 1911—1913 may be said to have laid the grounds for the dominating view that in densely and sparsely populated areas the poliomyelitis frequency is governed by the invisible immunization. In this connexion *von Pfaundler* (1928), *de Rudder* (1934), *Pette* (1936), *Fanconi* (1945) *et al.* all cite *Wernstedt's* works and investigations, which have also received support from experimental research (*Aycock* and *Kramer* (1930) on immunity among urban and rural populations to the poliomyelitis virus) as well as from the above-mentioned investigations of recent years in Sweden by *Olin* and *Heinertz* (1943, 1944).«

Es gibt jedoch, so fährt B. fort, eine Anzahl von Epidemien, wo die Poliomyelitis die soeben angeführte Morbiditätsverteilung vermissen lässt. Die Poliomyelitismorbidität kann infolgedessen nicht unter dem Einfluss immunisatorischer Kräfte stehen, denn eine Immunisie-

rung würde kaum irgendwelche Ausnahmen gestatten. »It may therefore,« heisst es weiter (S. 161), »be of interest to resort to the original source of the general view concerning the effect of invisible immunization on the distribution of poliomyelitis in dense and sparse populations, in urban and rural districts, viz. Wernstedt's investigations (1917) of the epidemics in Sweden during 1911—1913, and to inquire more closely into the grounds on which the author supports himself in this problem.«

Die von B. gegen die zitierten Untersuchungen vorgebrachte Kritik gipfelt darin, dass sie mit »misleading statistical methods« durchgeführt seien, wodurch die Resultate unrichtig geworden wären. B. legt seiner Studie statistische Erhebungen über die Poliomyelitis aus der ganzen zivilisierten Welt zugrunde. Das Untersuchungsmaterial ist mithin von imposanten Umfang. Unter diesen Umständen, und da die Frage der Methodik bei den hier erörterten immunologischen Morbiditätsuntersuchungen gewiss eine allgemeine und grundlegende Bedeutung für das Studium jeder epidemischen Krankheit besitzt, ist es selbstverständlich überaus wichtig, dass diese Frage eine eingehendere Durchprüfung erfährt als ihr bisher zuteil geworden ist.

Da es sich bei Untersuchungen dieser Art nicht um eine gewöhnliche einfache Krankheitsstatistik handelt, sondern um eine statistische Erfassung der *biologischen* Reaktionen, die durch Kontakt mit dem epidemischen Ansteckungsagens ausgelöst werden, sollten selbstredend keine anderen Personen den Gegenstand der statistischen Bearbeitung bilden als solche, bei denen ein derartiger Kontakt stattgefunden hat. Die Verwirklichung dieses Postulats liegt aber jenseits der Grenzen des Möglichen. Es bleibt daher nur das Bestreben übrig, jenem Ideal so nahe zu kommen wie irgend möglich, um auf diese Weise die Fehlerquellen im Schach zu halten, die in der Einbeziehung derjenigen Personen in die Statistik liegen, welche mit dem ansteckenden Agens nicht in Berührung gekommen waren.

Gewöhnlich hat man sich bei der Bearbeitung einschlägiger Fragen des für die amtliche Krankheitsstatistik bestimmten und hierbei auch zweckmässigen Verfahrens bedient, die Morbidität für die Bevölkerung des ganzen Reiches zu berechnen. Würde man aber bei einer Untersuchung, mit welcher der hier vorliegende Zweck verfolgt wird, dieses Verfahren anwenden, dann würde man doch offensichtlich das Material mit der grössten Anzahl von dem ansteckenden Agens unberührter Personen belasten — und damit mit der grössten Fehlerquelle in obiger Beziehung — wie es überhaupt in einem Lande möglich ist. Hierdurch würde eine richtige Lösung jener Frage *biologischen* Charakters (nicht von der Art der gewöhnlichen Krankheitsstatistik) kaum zu erreichen sein. Nur ausnahmsweise, unter speziellen Bedingungen, kann man erwarten, dass eine derartige Methode es den wirklichen immunologischen Verhältnissen gestatten würde, sich auf das Ergebnis auszuwirken. Was die Poliomyelitis anlangt, so ist dies z. B.

dann der Fall, wenn es sich um ein so anhaltendes und so allgemein das ganze Land umfassendes Auftreten der Krankheit handelt wie in den Jahren 1930—39 (s. unten), aus welcher Zeit des Material der *Olin-Heinertzschen* Untersuchung vom Jahre 1943 stammt.

Die besagte Fehlerquelle nach Möglichkeit auszumerzen und dadurch die Methodik zu verschärfen dürfte aber nur so möglich sein, dass man ohne Rücksicht auf geopolitische oder administrative Grenzen die Epidemiegebiete als solche möglichst abzugrenzen sucht, und dass man sich bei der statistischen Bearbeitung lediglich an die in derartigen Gebieten ansässige Bevölkerung hält. Auch diesem Verfahren haften naturgemäss Fehler an, denn es gibt selbstverständlich keine Möglichkeit, die Epidemiegebiete so zu begrenzen, dass innerhalb derselben nur vom Virus berührte und ausserhalb ihrer nur berührungsfreie Einwohner vorkommen. Einen anderen Weg, welcher näher an die ideale Methode heranführt, dürfte es jedoch nicht geben.

Eine ähnliche Betrachtungsweise ist am Platze um soweit möglich Fehlerquellen hinsichtlich der Beziehungen zwischen Morbidität und Bevölkerungsdichte zu vermeiden. Hierbei grundsätzlich Durchschnittswerte für so grosse administrative Einheiten, wie z. B. in unserem Lande die Regierungsbezirke (»län«), zu verwenden, wäre eine zu grobe und folglich zu unzuverlässige Methode. Nur in Ausnahmefällen und unter besonderen Umständen, wie beispielsweise bei den von mir mit diesem Verfahren untersuchten Bezirksgruppen (näheres hierüber in meiner Arbeit vom Jahre 1924, S. 301—302), kann der wirkliche Einfluss der verschiedenen Bevölkerungsdichte auf die Morbidität dann in der Statistik zum Vorschein kommen. Zu einem sichereren Resultat gelangt man indem man der Berechnung die in jedem Epidemiegebiet befindlichen kleineren, untereinander hinsichtlich der Bevölkerungsdichte verschiedenen Einheiten zugrunde legt, z. B. die einzelnen Gemeinden, oder durch individuelle Sortierung der Fälle nach Massgabe der an jedem Poliomyelitisort herrschenden Dichteverhältnisse.

Die umfassenden schwedischen Epidemien 1911—13 und 1930—39, die beide Gegenstand eingehender Untersuchungen über die Morbidität gewesen sind, verdienen zweifelsohne besondere Beachtung. Vergleiche derselben sind ferner geeignet, die in der Zwischenzeit erfolgte Morbiditätsverschiebung in Richtung auf höhere Altersklassen klar ersichtlich zu machen. Die bezüglich der Methodik wichtigsten Punkte werden daher im folgenden dargelegt werden.

In bezug auf die Gesamtmorbidität für Städte und offenes Land findet man bei Berechnung derselben an Hand der ganzen Reichsbevölkerung praktisch die gleiche Erkrankungszahl für Städte und Landbewohner in der kürzeren Periode 1911—13 (insgesamt 6778 Fälle). Was die Zehnjahrsperiode 1930—39 anlangt (insgesamt 8288 Fälle), so ergab dagegen diese Berechnungsweise eine fast doppelt so hohe Morbidität auf dem Lande gegenüber den Städten. Bei Zugrundelegung lediglich der Epidemiegebietsbewohner für die Berechnung erwies sich

indessen die Morbidität auf dem Lande in den Jahren 1911—13 als 3- bis 4mal so hoch wie in den Städten. Auch *Olin-Heinertz* konnten bei Anwendung dieses Verfahrens eine erhebliche Verdeutlichung der bereits bei Berechnung für das Reichsgebiet gefundenen Differenz zwischen Stadt und Land feststellen. Die Untersuchung, von der hier die Rede ist, bezog sich auf zwei grössere Epidemiegebiete während der Epidemien von 1936 und 1938. Bei der einen Epidemie war die Morbidität laut Befund dieser Autoren in den dünn besiedelten ländlichen Gegenden doppelt und in den am dünnsten bevölkerten dreimal so gross wie in Städten und stadtartigen Gemeinden. Bei der zweiten Epidemie betrug die Morbidität in den dünn besiedelten ländlichen Gegenden das Vierfache, in den am dünnsten bevölkerten nicht weniger als das Zwölfwache von der in Städten und stadtartigen Gemeinden.

Die vorstehenden Beispiele erscheinen geeignet, die Möglichkeiten der beiden Untersuchungsmethoden ins rechte Licht zu setzen. Indem man mit *epidemiologischen* statt *administrativen* Gebieten rechnet, bewirkt man, dass erwartungsgemäss die *biologischen* Reaktionen zwischen dem Virus und der von ihm berührten Bevölkerung in der Statistik eher zum Ausdruck gebracht werden.

Was die Morbidität in verschiedenen Altersklassen und ihre Beziehung zur Bevölkerungsdichte betrifft, konnte bei Bearbeitung der Epidemien auf dem Lande 1911—13 keine direkte Berechnung and Hand der Bevölkerung erfolgen, da die hierzu erforderlichen statistischen Unterlagen damals noch ausstanden. Infolgedessen mussten die Berechnungen ausschliesslich auf dem Poliomyelitismaterial fassen, welches nach Eintragung der Fallzahlen in eine Landkarte nach der Topographie derselben in 4 verschiedene Besiedlungskategorien, »dünnest«, »dünn«, »dicht« und »dichtest« bevölkerte Landstriche, eingeteilt wurde (s. beigelegte Abb. 1 und 2, die graphische Darstellungen von Tabelle XVIII in der Veröffentlichung vom Jahre 1924 sind). Dieses

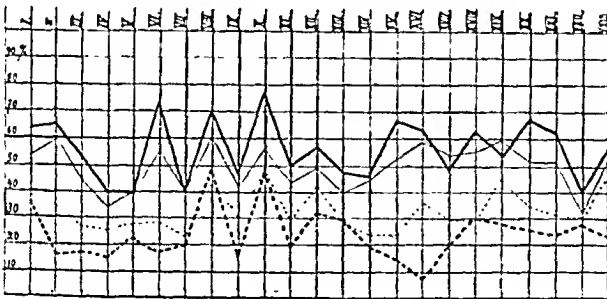


Abb. 1.

Das Morbiditätsprozent im Alter von 0—5 Jahren am Lande der verschiedenen Regierungsbezirke (»län«) auftretender Parasefälle der Epidemie 1911—1913 in Schweden (nach *Wernstedt*).

———— = dichtest, - - - - - = dünnest,
 - - - - - = dünn, - . - . - = dicht, = dünn bevölkertes Land.

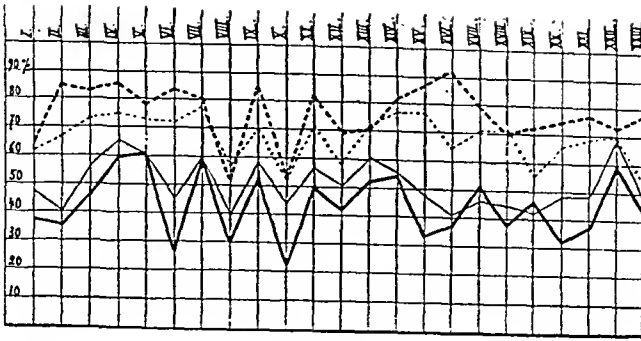


Abb. 2.

Das Morbiditätsprozent im Alter von 6 Jahren und darüber am Lande der verschiedenen Regierungsbezirke (»län«) auftretender Paresesfälle der Epidemie 1911—1913 in Schweden (nach Wernstedt).

———— = dichtest, ———— = dicht, = dünnest,
- · - · - · - = dünn bevölkertes Land.

Verfahren hatte den Vorteil der gänzlichen Befreiung von allen mehr oder minder unexakten Abgrenzungsversuchen sowohl der Epidemie- wie der Dichtegebiete, und damit der Vermeidung einmal der Einbeziehung in die Statistik der vom Virus unberührten Bevölkerung, sodann der Fehlplacierung, die dadurch zustande kommt, dass begrenzte Gebiete mit bekannter Durchschnittsdichte (z. B. Kirchspiele) in Wirklichkeit oft ein Gemisch von mehreren untereinander verschiedenen lokalen Dichtegebieten sind.

Olin-Heinertz konnten hingegen auf Grund der damals bereits vorliegenden amtlichen Statistik über die Verteilung der Landbevölkerung auf 4 verschiedene Dichtegruppen (A- bis D-Gemeinden) die Morbidität in diesen Gemeinden für die einzelnen Altersklassen direkt berechnen. Da ihre Resultate durchweg mit denen von 1911—13 übereinstimmen, ohne jedoch die gleiche Schärfe aufzuweisen, dürfte viel-

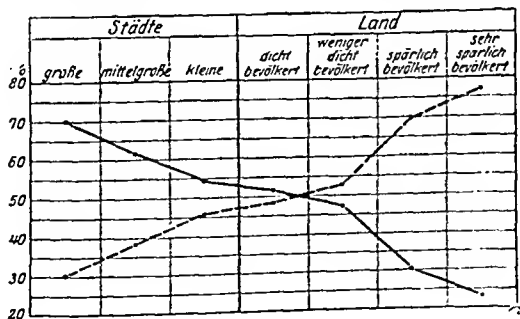


Abb. 3.

Die Kurven zeigen die Altersmorbidität der Paresesfälle von 1911—1913 in Schweden unter verschiedenen Kontaktmöglichkeiten. Diese sind am grössten in den Grossstädten und am kleinsten in dem dünnest (sehr spärlich) bevölkerten Land (nach Wernstedt).

———— = 0—5 Jahre, - · - · - · - = 6 Jahre und darüber.

leicht die ausschliesslich mit dem Poliomyelitis-material arbeitende Methode die sichersten Ergebnisse liefern.

Die gewonnenen Erfahrungen werden durch die beigefügten Kurven im einzelnen veranschaulicht. Wir sehen (Abb. 3) in bezug auf die Epidemiejahre 1911—13 (*Wernstedt*), wie die Morbidität in der jüngeren Altersgruppe (0—5 Jahre) von einem Minimum in den am

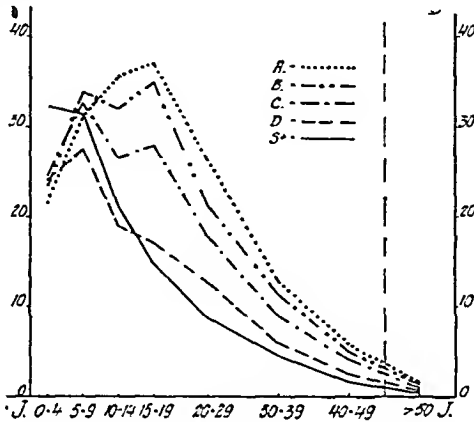


Abb. 4.

Morbidität der verschiedenen Altersklasse für die Landgemeinden A—D und für die Städte bei den Poliomyelitis-epidemien in Schweden 1930—1939. Jährlicher Mittelwert auf 100,000 Einwohner (nach *Olin-Heinertz*).

dünnsten besiedelten Landbezirken stetig ansteigt, um in den Grossstädten zu kulminieren. Die entsprechende Registrierung für die jüngste Altersgruppe (0—4 Jahre) aus der Periode 1930—39 (*Olin-Heinertz*) macht, wie aus Abb. 4 hervorgeht, einen im grossen und ganzen ähnlichen Verlauf ersichtlich. So ist die Morbidität auch hier in den am dünnsten besiedelten Landbezirken (A) am niedrigsten und in den Städten (St) am höchsten. Was die höheren Altersstufen angeht, so sind diese nicht wie in der *Wernstedtschen* Untersuchung in einer Gruppe zusammengefasst sondern auf mehrere verteilt. Jede höhere Altersgruppe (die Altersgruppen 5—14 Jahre noch nicht ganz regelmässig) zeigt indessen den für die höhere Altersgruppe in der *Wernstedtschen* Kurve charakteristischen Rückgang der Morbidität mit steigender Bevölkerungsdichte. Die Kurven von *Olin-Heinertz* (Abb. 4), verglichen mit derjenigen in Abb. 5 für die Epidemie 1911—13, machen überdies klar ersichtlich, dass seit dieser Epidemie in unserem Lande eine Verschiebung der Morbidität in Richtung auf höhere Altersstufen stattgefunden hat.

Aus den Untersuchungen geht mithin eindeutig hervor, dass es eine Präzession bei der Poliomyelitis gibt. *Bertenius* hat sich grosse Mühe gegeben, das Gegenteil zu beweisen. Seine Beweisführung krankt daran, dass B. zwischen Präzession und der soeben besprochenen allgemeinen Altersverschiebung der Morbidität nicht scharf unterscheidet. Um seine Anschauung zu stützen, verweist B. ferner auf eine Zu-

sammenstellung von Infektionskrankheiten, die nur zum Teil wie die Poliomyelitis akute, spezifische, epidemische und immunisierende Erkrankungen sind. Der Rest besteht aus teilweise chronischen, teilweise nicht epidemischen, teilweise nicht spezifischen und teilweise nicht immunisierenden Infektionen. Es ist dies folglich ein Material, das sich zu Vergleichen mit der Poliomyelitis wenig eignet. Die Schluss-

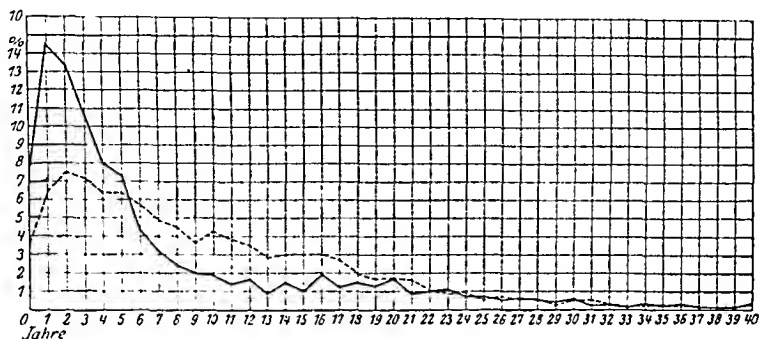


Abb. 5.

Morbidity der verschiedenen Altersklassen per Prozent sämtlicher Parasefälle der schwedischen Poliomyelitisepidemie 1911—1913 (nach Wernstedt).

———— = Städte, - - - - - = Land.

folgerung ist (S. 151) »that poliomyelitis is a disease which significantly differs from the 17 other infectious diseases represented here. All of these have been found to follow the laws of the precession of thorough immunization.«

Merkwürdigerweise stellt man jedoch in den Tabellen fest, dass eben jene Krankheiten, welche wie Masern, Scharlach und Diphtherie bei einem Vergleich mit der Poliomyelitis am wichtigsten sind, denselben Charakter (keine Präzession) aufweisen wie letztere. B. meint indessen diese Tatsache mit der Bemerkung aus der Welt schaffen zu können, dass sie durch »the prophylactic therapeutic and hygienic measures at the disposal of urban and rural districts during these years« (S. 123) bedingt sei — eine Behauptung, deren Berechtigung erst einmal bewiesen werden soll!

»The deficiencies of the immunity or possibly its complete absence,« sagt B weiter (S. 192), »is also illustrated by the significant displacement towards higher ages,« welches seit der Epidemie 1911—13 stattgehabt hat. Auf den ersten Blick mag es vielleicht so scheinen. Hand in Hand mit dieser Altersverschiebung ist aber eine für die Ansteckungsverbreitung äusserst wichtige Veränderung in unseren Umweltverhältnissen vonstatten gegangen, die B. vollständig ausser acht gelassen hat (diese Veränderung wird jedoch in meinen Untersuchungen vom Jahre 1939 erörtert). Ich meine die revolutionierende Entwicklung unserer Verkehrsmittel seit 1911—13, die ebenfalls immer zunehmende »Flucht vom Lande« nach den Städten, und ferner die

immer mehr fortschreitende Urbanisierung der Landgemeinden. Hierdurch ist die Einwohnerschaft der Städte seit einer Reihe von Jahren in erheblichem Umfang von Landbewohnern, namentlich solchen jugendlichen Alters oder in mittleren Lebensjahren, durchdrungen worden. Infolge der ausserordentlichen Erweiterung der Verkehrsmöglichkeiten zwischen Land und Stadt ist die Landbevölkerung ausserdem noch in näheren Kontakt mit den Städten gekommen, und dadurch wesentlich grösseren Ansteckungsgefahren ausgesetzt worden als nur ein paar Jahrzehnte früher. Es dürfte auf der Hand liegen, dass diese Nivellierung der Gegensätze zwischen Stadt und Land sich kaum anders auswirken kann als in einem gewissen Ausgleich der früheren Divergenzen in bezug auf die Altersmorbidity der betreffenden Gruppen. Die Verschiebung der Poliomyelitis-morbidity in Richtung auf höhere Altersstufen dürfte somit keineswegs gegen eine invisible Immunisierung sprechen, sondern im Gegenteil ein beachtenswerter Beleg für den Bestand derselben sein. Unter allen Umständen darf die durchgreifende Veränderung, die seit dem zweiten Jahrzehnt unseres Jahrhunderts in bezug auf die gegenseitige Berührung der Stadt- und Landbevölkerung vorstatten gegangen ist, bei einer *vorurteilsfreien* Diskussion über das veränderte Auftreten der Poliomyelitis in Stadt und Land, in jüngeren und höheren Altersstufen, keinesfalls ansser acht gelassen werden.

Für das Bestehen der invisiblen Immunisierung spricht noch eine andere, von B. ebenfalls stillschweigend übergangene Tatsache — die Neigung der Poliomyelitis, nicht Jahr für Jahr in *epidemischer* Form in demselben Gebiet aufzutreten, in dem sie wenige Jahre zuvor grosse Verheerungen angerichtet hatte. Ein Blick auf die von mir zusammengestellte Karte über die Epidemiegebiete in unserem Lande während der Jahre 1905, 1911, 1912 und 1913 (s. Erg. d. inn. Med. u. Kindhk. 1924, S. 349—350) dürfte auf recht überzeugende Weise lehren, dass wir es hier nicht mit dem Spiel des Zufalls zu tun haben, sondern mit einer in immunisatorischen Verhältnissen wurzelnden Gesetzmässigkeit.

Im Anschluss an die obigen Ausführungen möchte ich noch einige spezielle Einwände von B. richtigstellen.

1. Gegen meine Angabe, dass die höchste Morbidity, welche in den grössten Epidemiegebieten 1911—13 beobachtet wurde, die Landgemeinden betraf, wendet B folgendes ein: Unter der Voraussetzung, dass diese Schlussfolgerung »has reference to rural communes in general« in den betreffenden Regierungsbezirken, kann ein solcher Schluss nicht gezogen werden, da sich die Untersuchung nur auf einen verschwindend geringen Teil der Gemeinden in den gesagten Bezirken bezieht. Etwas derartiges habe ich aber nicht vorausgesetzt, wovon sich jeder leicht überzeugen kann. B. stellt also einer unbestreitbaren Tatsache eine wirklichkeitsfremde Konstruktion entgegen. Der wissenschaftliche Wert einer solchen Kritik ist gleich Null. Dasselbe könnte man von den übrigen Punkten sagen.

2. Meine Angabe, laut welcher die Morbidity in Kleinstädten höher ist als in mittelgrossen Städten, und in letzteren höher als bei Epidemien in Grossstädten, soll nach B. »statistically unsupported« sein. Das angewandte Ver-

fahren, nur Städte mit mindestens 20 Poliomyelitisfällen zu berücksichtigen, »must give highly-deceptive results«, da hierdurch nur die Hälfte der mittelgrossen Städte, und nicht einmal 10 % der Kleinstädte, von der Statistik erfasst werden. B. unterlässt es aber, zu erwähnen, dass sich die diesbezügliche Untersuchung, wie ausdrücklich gesagt, nur auf Städte erstreckte, in denen die Poliomyelitis eindeutig *epidemisch* auftrat. Um in dieser Beziehung mit einem reichlichen Sicherheitsmarginal zu arbeiten, zog ich die Grenze für ein epidemisches Auftreten bei mindestens 20 Fällen. Auch in diesem Punkte ist die Kritik folglich bloss einen Schlag in die Luft.

3. Meine Angabe, dass die Morbidität auf dem Lande in den 4 Regierungsbezirken mit grösster Bevölkerungsdichte niedriger war als in 4 anderen schwer heimgesuchten Bezirken mit geringerer Bevölkerungsdichte, und dass dies nicht dagegen spricht, dass in dicht besiedelten Gegenden eine niedrigere, in dünn besiedelten eine höhere Morbidität vorherrscht, sucht B. ebenfalls damit abzufertigen, dass er die Erörterung auf eine andere Frage lenkt. »If Wernstedt had calculated,« sagt B. (S. 164), »the paresis morbidity in the towns as well as in the four most densely populated läns,« würde er gefunden haben, dass die Morbidität in den Städten in den untersuchten Bezirksgruppen dieselbe war wie auf dem Lande, was nach B. gegen die von mir verfochtene Ansicht sprechen soll.

Was B. unter Zuhilfenahme einer umständlichen, völlig auf der mangelhafteren Untersuchungsmethodik fussenden Erhebung hierzu anführt, kann doch natürlich nicht die Tatsache umstossen, dass sich die Morbidität *auf dem Lande* in den untersuchten Bezirksgruppen so verhält wie von mir angegeben, und dass dieses Faktum, wie ich behaupte, nicht mit der von mir verfochtenen Ansicht über die Beziehung zwischen Morbidität und Bevölkerungsdichte in Widerspruch steht.

Zusammenfassend dürfte zu sagen sein,

- 1) dass *invisible Immunisierung und Präzession* offenbar für die *Poliomyelitis charakteristische Erscheinungen* sind,
- 2) dass die von Bertenius vorgebrachte Kritik als *unhaltbar abzulehnen* ist, und
- 3) dass die *Methodik, welche B. seinen Poliomyelitisstudien zugrunde gelegt hat, viel zu grob ist, als dass sie in grösserem Umfang richtige Ergebnisse liefern könnte.*

Es kann nämlich nicht so sein, dass Aufklärungen über Rückwirkungen des Poliomyelitisvirus auf die Bevölkerung am sichersten dadurch erhalten werden, dass in das Untersuchungsmaterial die grösstmögliche Anzahl Personen einbezogen werden, die *nicht* mit dem Poliomyelitisvirus in Berührung gekommen waren. Ebenso verfehlt ist es, beim Studium der Bedeutung der Bevölkerungsdichte für die Ansteckungsverbreitung und Morbidität mit *nivellierende* Durchschnittswerten für so grosse Verwaltungseinheiten wie Regierungsbezirke zu manipulieren statt mit Werten für kleinere, innerhalb dieser Gebiete differenzierte Einheiten, wie z. B. die einzelnen Landgemeinden.

LITTERATUR

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ON THE PROBLEM OF POLIOMYELITIS

Reply to Professor W. Wernstedt's criticism.

By Bertel S:son Bertenius.

(Received for publication March 12th 1948.)

The Editorial Management of *Acta Pathologica et Microbiologica* has kindly given me an opportunity of acquainting myself with Professor Wilhelm Wernstedt's critical review of my work »On the Problem of Poliomyelitis«, and I am gratefully taking the opportunity to submit some comments and refutations of this criticism.

Wernstedt's view is of course correct that a statistical investigation dealing with the biological reaction between an epidemic virus and its human victims should so far as possible only take in those persons who have come into contact with the virus. However, the difficulties associated with this, as is also pointed out by Wernstedt, lie in accomplishing this in practice without influence from too many sources of error.

In his study of this problem during the great poliomyelitis years of 1911—1913 in Sweden, Wernstedt considered that he could attain the best results by confining himself exclusively to the areas ravaged by the disease and studying within these the morbidity in relation to the population within the smallest administrative units, the individual rural and municipal communities. According to Wernstedt, this furnished the best possible picture of the susceptibility to the disease in the locality in question and consequently of the immunity level.

Closer consideration, however, shows that there are several objections to be made against this method when such a disease as poliomyelitis is concerned. For instance, how can it be determined with certainty which communities are *not* infected? Since Wickman's investigation of the poliomyelitis epidemic of 1905 in Sweden it has been generally considered that the principal spreaders of the infection are not the manifest cases of paresis, which are estimated by our foremost authors not to amount to more than 1 per cent of the trans-

mitters, but the abortive cases and healthy virus carriers, which accordingly make up 99 per cent. With Wernstedt's view of the dissemination of the disease by direct contact it is difficult to believe that all these potential spreaders of infection are only to be found in communities with manifest poliomyelitis cases. Nothing can surely prevent them from moving freely to adjacent communities, and so on. We cannot therefore ignore the fact that many and probably all communities adjoining an »infected« community are to be regarded as infected areas. In such circumstances the relative morbidity in the individual communities, whether high or low, seems to be a chimera, for the only thing we know with certainty is that the risks of infection are not limited or restrained by the communal boundaries. Under such conditions it seems impossible to define the epidemic area with any exactness.

Wernstedt's method therefore seems to me to involve greater sources of error than that of estimating the morbidity in relation to the population within larger areas, e. g. a whole *län* (county). This is especially true in times of epidemics when all *läns* are attacked.

Therefore, when it can be shown from Wernstedt's material of paresis for the years 1911—1913 that the relative frequency of this disease is as high in the towns as in the rural districts of the four *läns* most attacked, this fact seems to be of quite as great a significance for the illumination of the morbidity risks and with them of the state of immunity in densely and sparsely populated areas as the fact established by Wernstedt that the relative morbidity in the rural districts in the four most ravaged *läns* was higher than in the rural districts in the four most densely crowded ones. Three of the former ones even belonged to the densely populated half of the Swedish counties. It is likewise remarkable that during the years 1911—1913 the four most densely and the four most thinly populated *läns* show practically the same incidence of paresis, or 2.7 in the former and 2.9 in the latter per 10,000 inhabitants and year, although the population of the towns and populous localities form a considerably larger relative proportion of the population in the former than in the latter.

In my criticism of Wernstedt's investigation of the risks of contracting poliomyelitis in large cities (above 100,000 inhabitants), medium-size cities (between 10,000 and 100,000 inhabitants) and small towns and town-like communities (with below 10,000 inhabitants) my objection is directed against a method which, as an expression of »a real epidemic appearance« of poliomyelitis, sets up the same minimum demand for the morbid frequency, viz. 20 cases, for all towns irrespective of whether they have over 100,000 inhabitants or under 10,000.

The method employed by Wernstedt thus involve a several times higher minimum demand for the morbid frequency per 1,000 inhabitants in the medium-size towns than in the large cities, and a

further manifold higher minimum demand for the frequency of the disease per 1,000 in the small towns and town-like communities than in the medium-size towns.

Under such conditions it is not surprising that all the three large towns can fulfil the relatively small minimum conditions set up for them, or that only 11 of 27 medium-size towns and not more than 10 of the close upon 200 small towns and town-like communities (with at least 1,000 inhabitants) can meet the considerably higher minimum demand placed upon them.

With the method used it is also self-evident that the mean value for the paresis frequency of the epidemics per 10,000 inhabitants will be the lowest in the large towns, higher in the medium-size towns, but many times higher in the small towns and small communities — N.B. in a few though intensely attacked ones. But I do not believe that the result of a comparison between mean values based on such different premises can have any scientific value.

On page 7 Wernstedt writes that my tables (see Tables 56 and 57) would indicate that scarlatina, diphtheria and morbilli exhibit the same character as poliomyelitis in respect of precession. This interpretation is doubtless due to a misunderstanding based on the fact that between the periods 1911—1920 and 1931—1938 all these diseases do not show any positive effect of the precession. In the case of the three mentioned first this fact does not depend upon deficient power to produce immunity, which seems evident from their mortality diagrams, Figs. 10—17 and 38—40 in my work. Annual period after annual period the highest mortality per 100,000, and consequently also no doubt the morbidity, are to be found in the as yet not thoroughly immunized youngest ages. Compare with these the diagrams for the poliomyelitis mortality, Figs. 22—25, annual period by annual period. The difference is not difficult to detect.

The absence of the precession effect in diphtheria may doubtless be explained by the fact that during the thirties that disease was in process of being eradicated in Sweden. In spite of increased opportunities for contact the bacillus carriers were so few in number that it took a longer time than 20 years earlier for the younger, the non-immune ages to become exposed to infection from them.

That scarlatina and morbilli did not show an increased relative share in the mortality of the age group 0—5 years during the thirties is, I consider, a consequence of improved hygienic, prophylactic and therapeutic facilities. This assumption seems to me to be supported by the fact that the mortality in these diseases — though not therefore with equal certainty the morbidity — diminished much more rapidly in the towns, where these facilities are available to a greater extent than in the rural districts. For instance, the scarlatina mortality in the youngest age group diminished from 39.7 (1911—1920) to 5.4 (1931—1938) in the towns, from 23.0 to 8.8 in rural districts, and

the morbilli mortality from 74.5 to 11.5 in the towns and from 44.0 to 11.0 in the rural districts, all per 100,000 children per annum.

For poliomyelitis, on the other hand, it is found that the mortality (for the same age group and the same number of children) fall from 7.7 to 3.0 in the towns but from 9.8 to 1.7 in country districts. As is evident from this, the decline in poliomyelitis mortality in the age group 0—5 years was greater in the rural districts. Probably this was not due to either reduced risks of infection or to improved hygienic or medicinal factors, which have not hitherto proved at all able to reduce the frequency or mortality of poliomyelitis. If such factors had exercised an influence, this ought surely to have come to stronger expression in the towns.

The powerful fall in the poliomyelitis mortality during the thirties in the ages of infancy and early childhood, which also corresponds to a distinct regression in the paresis frequency in the same ages (in Sweden) — see Table 49 A and B, is therefore in glaring contrast to all the numerous biological reactions which during this period, with its considerably increased risks of contact and infection, must have taken place between the poliomyelitis virus and the ages in question, both in towns and in the country. *If the p. virus had been homopathogenic in the same sense as those of scarlatina, diphtheria and measles, a powerfully pronounced precession could hardly have failed to appear in poliomyelitis during the thirties, a distinctly more powerful one than the precession effect of 20 years earlier.*

What Wernstedt considers to prove the presence of precession in poliomyelitis is that during the 1911—1913 epidemics he found that the age group 0—5 years had its highest relative morbidity in the towns while its relative morbidity declined in the country-side according as the density of the population decreased, the minimum being reached in the most sparsely populated districts. Wernstedt has obtained support for his view from Olin-Heinertz's investigations in Sweden for the years 1930—1939. These show the same trend. However, it seems to me that such a trend and parallelism between the relative poliomyelitis morbidity in the 0—5 years group and the density of population in the localities only signify that there is a positive correlation here between these two factors. The correlation as such reveals nothing as to its cause. If the cause of the correlation had lain in different states of immunity in the localities and had hence been an effect of precession, this ought to have brought about — as was just pointed out — an entirely different effect of the precession during the thirties. We ought then to have had the right to expect quite different and *increased* relative morbidity rates in the ages of infancy and early childhood, the least immune, than 20—25 years earlier. Now these relative paresis figures only reach about 50 % (see Table 49 A and B), in towns and rural districts, of their value during 1911—1913. This fact seems impossible to reconcile with the view that the age distribu-

tion of the poliomyelitis morbidity is conditioned by immunological laws.

On page 8 Wernstedt writes that I have completely ignored the great importance for the transmission of infection that the ever-increasing inter-communications had after 1911—1913. In reply I would refer to what I have written on pages 109 (14th line from bottom), 122 (17th line from bottom), 123 (9th line from bottom), 124 (11th line from bottom), 147 (21st line from bottom) and 151 (1st line), and would further stress that Chapter 6 (on the problem of precession) is wholly built up on a comparison of the two periods of time with their different risks of contact and consequently different risks of infection, based just upon the different intensity of communications that characterized these periods.

If the increased invasion of the towns by young and middle-aged people during the thirties had, as Wernstedt thinks, actually increased the number of the vulnerable in these ages in the towns and had thus also increased the general poliomyelitis morbidity of the towns, so that an equalizing tendency had in this way been established between the rural and the urban risks, this ought to have become visible in Table 49 A and B, page 108, showing the paresis morbidity in different ages in towns (and town-like communities) and in rural districts during the years 1911—1913 (according to Wernstedt's paresis data) and during the period 1930—1939 (according to Olin-Heinertz's paresis figures). These tables, however, do not lend any support to such a view. During 1911—1913 the share of the towns in the paresis cases (estimated on the morbidity per 100,000 inhabitants in the various age groups) was 44.5 % as against 55.5 % for the country districts. During 1930—1939, on the other hand, the corresponding figures were 41.3 % and 58.7 %.

During the thirties all age groups over 15 years certainly increased their relative morbidity compared with their morbidity during 1911—1913, but they still kept the same mutual morbidity risks as between towns and rural districts as they had during the epidemics of 1911—1913, *i. e.* the incidence of paresis is about double as high in the same age-group (per 100,000 inh.) in the rural districts as in the towns. This does not, either, argue in favour of any equalizing tendency between the risks of contracting the disease in towns and country-side.

When Wernstedt writes that the increased morbidity risk observed for the older ages co-incidentally with the increased communications during the thirties does not argue against an invisible immunization but on the contrary gives powerful support to its existence, I do not understand his line of reasoning. He also considers that poliomyelitic epidemics have a rather thorough immunizing effect in the localities attacked. In this respect I refer to his view of the 1905 epidemic, see below. If all the epidemics occurring in Sweden after 1905 had possessed the same ability, *the cumulative result during the thirties ought*

presumably to have been an appreciable reduction of the susceptibility in the older ages instead of an increase.

»Für das Bestehen der invisiblen Immunisierung spricht noch eine andere, von B. ebenfalls stillschweigend übergangene Tatsache — die Neigung der Poliomyelitis, nicht Jahr für Jahr in *epidemischer* Form in demselben Gebiet aufzutreten, in dem sie wenige Jahre zuvor grosse Verheerungen angerichtet hatte. Ein Blick auf die von mir zusammengestellte Karte über die Epidemiegebiete in unserem Lande während der Jahre 1905, 1911, 1912 und 1913 (s. Erg. d. inn. Med. u. Knndhlk. 1924, S. 349—350) dürfte auf recht überzeugende Weise lehren, dass wir es hier nicht mit dem Spiel der Zufalls zu tun haben, sondern mit einer in immunisatorischen Verhältnissen wurzelnden Gesetzmässigkeit.« writes Wernstedt on page 9.

It is true that I have not dealt with this frequently observed phenomenon in the epidemiology of poliomyelitis, but my investigation has as a principle only taken up problems for which the official statistics has been able to supply material. However, it should not be overlooked that although the localization of epidemic foci in our country during the years enumerated lends support to Wernstedt's view that a determinate process of immunization has made itself felt here, other investigators, *e. g.* Bergstrand in Sweden (Nord. Med. No. II, 1939, p. 1276) and Lumsden in the U. S. A. (South. Med. J., May 1938, p. 472), have adduced facts that do not so clearly point in the same direction. Lumsden, the Medical Director of the U. S. Public Health Service, writes concerning this: »The experience of Mississippi« (for the years 1934—1937)« was similar to that observed previously in other sections of the U. S. There have been instances of *high incidence rates* in one region or locality for two or more successive years, but, as a rule, communities after having a severe outbreak have low incidence rates for one or two or more years thereafter«. *It therefore appears as though we are not concerned here with any immutable immunological conformity to law.* We can therefore hardly exclude the influence here of other at present unknown factors.

ON THE SO-CALLED NASAL GLIOMAS (ENCEPHALOCHORISTOMA NASO-FRONTALIS)

By *Lennart Zettergren.*

(Received for publication March 16th 1948.)

»The gliomas are tumours derived from the cells which constitute the supporting tissue of the nervous system«. The genuine eye ground gliomas will be seen to conform to this definition of *Russel Brain's*, when considering the connection between the brain and the visual organs from an evolutionary point of view. On the other hand, the occurrence of so-called gliomas in the nasal region seems to be less easily explained. Since the genesis of these tumours is still being discussed, it seems appropriate here to give an account of a case of this kind which has been subjected to a close study.

A boy, two months old. Since birth, a tumour at the nasal root to the left of the median-line. No noteworthy growth of the tumour. The child's development otherwise normal. At examination, a tumour, the size of a hazel-nut, of a soft consistency and movable, was found on the left side of the nasal root. At operation, the tumour was extracted in toto and seen to be fairly well-delimited, having also a thin stalk, rich in vessels, extending down towards the nasal bone. No connection with the cranial cavity was noted. Unfortunately, no röntgenological examination was made to ascertain the possible presence of a sutural dehiscence.

Histological examination: The formation in question, which was approximately the size of a hazel-nut, was found to consist histologically of neuroglial tissue rich in vessels, which was not demarcated from the skin but apparently forced in between the collagenic bundles right out to the epidermis (Fig. 1). A comparatively compact glial web extended between the tumour cells which, as a rule, had a sparse amount of cytoplasm and mostly rounded or oval nuclei (Fig. 2). No mitoses were observed. Here and there, particularly in the proximity of the coarser vessels, relatively large glia cells were seen with several,

usually peripherally situated, nuclei (Fig. 3). In some places there gliogenous giant cells were elongated, their nuclei lying in a row, one after the other. Also isolated cells were noticed which, after Nissl staining, had a granular basophile cytoplasm and round nuclei, poor in chromatin, with a generally central, large, nucleolus (Fig. 4a). In preparations impregnated with silver after Bielschowsky, these cells were found to be supplied with two straight processes or more which occasionally contained neurofibrils, proving their nature of ganglion cells (Fig. 4b). No medullary sheaths were ascertainable. *Pathologic-anatomical diagnosis: Encephalochoristoma.*

Discussion.

To the pathologist the so-called nasal gliomas represent exceedingly rare and surprising findings. However, a number of cases of such tumours are reported in the literature. In view of the fact that these tumours may be localized either subcutaneously on the ridge of the nose or in the nasal cavity, in rare instances even in both these places, it seemed justifiable in the following compilation of cases from the literature to pay regard to this varying localization in order to discern, if possible, the differences that may exist between the various groups.

Table 1.
1. Intranasal gliomas.

Year	Author	Age of patient at operation	Sex	Exact localization of tumour	Pathologic-anatomical diagnosis
1905	Clark	10 weeks (congenital)	♂	High up in septum in left nasal cav.	Glioma
1820	Anglade & Philip	3 days (congenital)	?	Right nasal cav.	Glioma
1926	Terplan & Rudofsky	13 years	♀	Left cavity of the ethmoid bone	Glioma? (Some features resembling neurinoma)
1927	Guthrie & Dott	46 years	♂	Left nasal cav.	Nasal polyp with neuroglial islands
1929	Tobeck	40 years	♂	Right maxillary cavity and ethmoidal cavity	Glioma

2. Extranasal gliomas.

Year	Author	Age of patient at operation	Sex	Exact localization of tumour	Pathologic-anatomical diagnosis
1900	Schmidt	10 weeks (congenital)	♂	Right nasal root	Glioma
1909	Süssenguth	11 days (congenital)	♂	Slightly to the left of nasal root	Glioma
1920	Berblinger	3 months (congenital)	♂	To the left of nasal root	Glioma or fibroglioma (occasional cells resemble ganglion cells but lack nucleoli)
1937	Eigler	10 years (about 6 yrs)	♂	Right part of ridge of nose	Glioblastoma
1924	Rocher & Anglade	? (congenital)	?	To the left of nasal root	Fibroglioma
1924	Rocher & Anglade	10 months (congenital)	?	To the right of nasal root	Fibroglioma (isolated nerve cells)
		18 months (congenital)	?	To the left of nasal root	Fibroglioma
1946	Bratton & Robinson	1 month (congenital)	♀	To the left of nasal root	Glioma
1948	Author's case	2 months (congenital)	♂	To the left of nasal root	Encephalo-choristoma

3. Extra- and intranasal gliomas.

1905	Clark	2 years (congenital)	♂	Upper part of ridge of nose and left nasal cavity	Glioma
1924	Rocher & Anglade	6 months (congenital)	?	Slightly to left of nasal root and left nasal cavity	Fibroglioma (Ependyma-coated canal. Neuroblastlike cells)

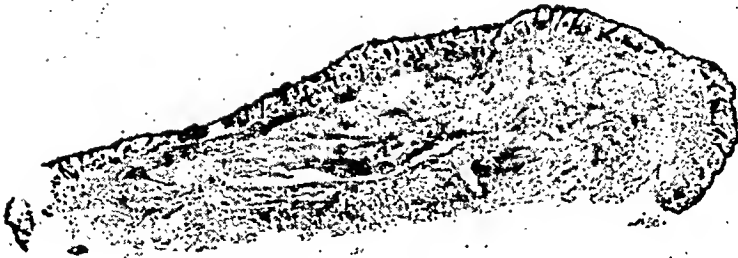


Fig. 1.

Survey picture of the tumour. The light spots consist of glial tissue.

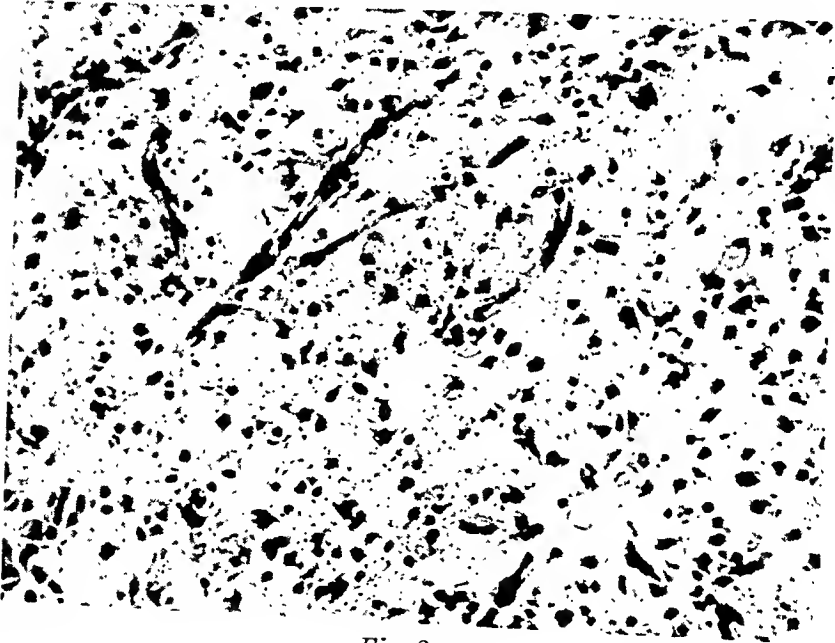


Fig. 2.

The tumour consists of fairly uniform glia cells and a connective tissue stroma rich in vessels. A compact glial web extends between the cells.

As is evident from the above compilation of cases, the subcutaneous nasal gliomas are all congenital, and the patients have been submitted to operation a comparatively short time after birth. The case of glioblastoma published by *Eigler*, however, represents an exception, involving a boy of ten years whose tumour was reported to have appeared »a few years« prior to the surgical intervention in the form

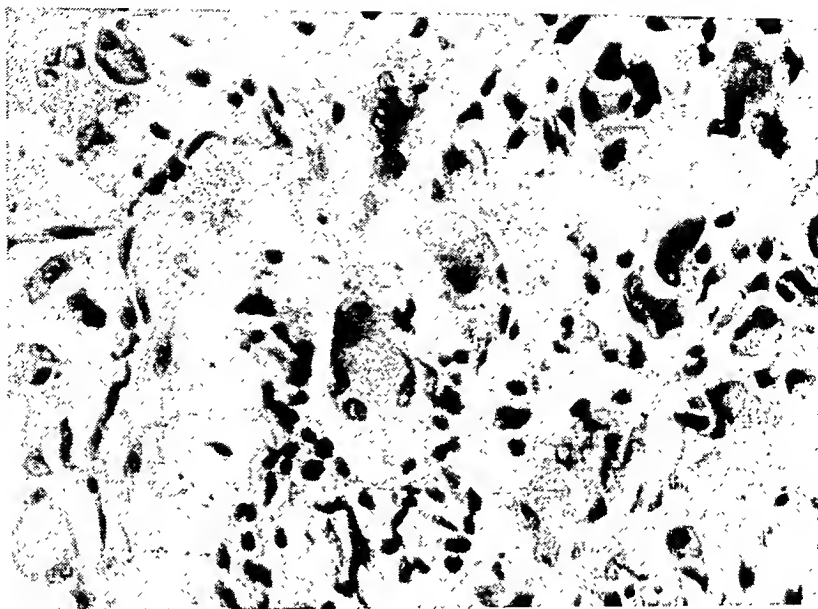


Fig. 3.

A section of the tumour with abundance of polynuclear, gliogenous giant cells.

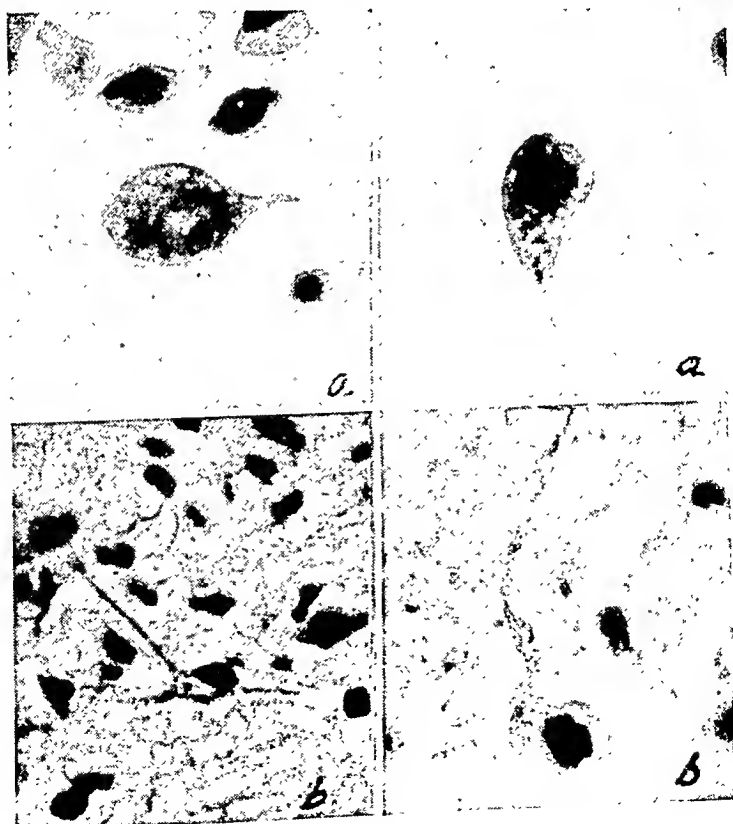


Fig. 4.

- a. Large pear-shaped ganglion cells with Nissl bodies and nuclei containing big nucleoli. Nissl staining.
- b. Similar cells in Bielschowsky neurofibril staining, showing indications of neurofibrils in dendrites.

of a small spot on the ridge of the nose. On the other hand, as regards the gliomas of the nasal cavity, in three of the cases the tumour was actually not manifest until the patient had reached a higher age (viz., 13, 40 and 46 years). Still, here also the congenital nature of these tumours may be questioned. The fact that a congenital intranasal tumour of the kind dealt with here does not show any particularly acute symptoms until the patient has advanced in years may, conceivably, be due to an edematous swelling of the tumour, *e.g.*, in connection with an acute rhinitis. Thus, the observation that, apart from the aforementioned exceptions, all the cases described in the literature of so-called nasal gliomas are definitely congenital — revealing in their histological structure, on the whole, fair conformity with the tumours of the three older patients — offers some support for the assumption that the tumours also in the three latter cases were congenital. Finally, it seems appropriate to apply the same genetic viewpoints to these histologically analogous formations. However, in principle, this would presuppose the congenital nature of all the tumours in these cases.

As to the exact localization of the so-called nasal gliomas, in five cases in the present compilation they were found to the right of the median line, and in ten to the left. Further, it is noteworthy that in the majority of the cases the subcutaneous tumours are situated at the nasal root. This observation is of great significance to the discussion of the genesis of these tumours.

The opinions stated in the literature concerning the genesis of the so-called nasal gliomas are somewhat divergent. Some authors (*Schmidt, Clark, Berblinger*, and others) interpret them as marginal severances from the anterior cerebral vesicle that have taken place at an early embryonic stage. Others again (for instance, *Süssenguth* and *Tobeck*) place them in connection with the strong embryonic development of the rhinencephalon and the construction of the primitive olfactory cavities. The possibility that these formations might constitute the one-sided development of teratomas is pointed out, among others, by *Schmidt* and *Clark*. However, at the same time, they repudiate this, since teratomas consisting *only* of nervous tissue are never seen in other connections.

In the author's opinion, the first of the three theories reported here is the most well-founded. As previously mentioned, the majority of gliomas of the ridge of the nose are localized to the nasal root. This region of the face has long been known to pass through a complicated embryonic development where an abnormal course may sometimes result in the occurrence of a regular encephalocele nasofrontalis. The studies reported by *Holl* are of great value for an understanding of the origin of the encephalocele. Thus, *Holl* showed that a connective tissue protuberance issues from the dura in normal cases, on the border between the horizontal ethmoid plate and the frontal bone. This pro-

tubercle fills the entire fossa supranasalis, i.e., the small triangular hollow lying behind the nasal bones in the upper part of the cartilaginous wall of the nasal capsule. There is a cord running from this protuberance in the sagittal margin between the nasal bones and the cartilages right down to the tip of the nose. The protuberance itself constitutes the mesenchymal rudiment of the nasal process of the frontal bone. Thus, a bone capsule later develops on its surface. The intracranial part of the canal filled by the protuberance of connective tissue lies right inside the foramen coecum, immediately in front of the crista galli. An explanation of the predominantly left-sided localization of nasal gliomas may, perhaps, be found in the generally asymmetric leftward course of this canal. If a deficient development of the dura takes place in this particular region, it will involve a defect in the aforementioned dural protuberance that projects down through the foramen coecum, with the consequent possibility of a prolapse of a cerebral part through this opening. Further support of *Holl's* supposition is offered by *Muhr*. The latter was in a position to prove on autopsy in a case of encephalocele that a solid protuberance of cerebral substance, which appeared between the nasal bones and the nasal cartilage capsule, was connected with the cranial cavity at the anterior edge of the ethmoid plate.

In the author's opinion, in principle, the so-called nasal glioma is an occurrence similar to that of an encephalocele nasofrontalis. The difference is merely that the encephalocele develops at a late embryonic stage when the skull has already ossified, while in the case of a nasal glioma the nervous tissue is already established when the ossification takes place. This theory derives support from the fact that *Rocher & Anglade* were able to ascertain, in at least one of their cases of nasal root glioma, how one stalk of the tumour penetrated deeper. From this stalk a clear fluid flowed that was found on chemical examination to contain sugar. Thus, it could definitely be assumed to consist of cerebrospinal fluid. The patient died of symptoms indicating a meningitis. A similar protuberant continuation of the tumour down into deeper regions is to be noticed in the case published by *Eigler*, as well as in my own case.

The histological structure of the so-called nasal gliomas, should also, it seems, be of considerable significance to the discussion regarding their genesis. Provided, as previously assumed, that they constitute severed cerebral parts, it would be expected that they should be histologically traceable in the tissue components forming the brain at the stage when the severance happened, i.e., apart from glial tissue, also nerve cells that develop at a very early embryonic stage. However, this does not always occur. While neuroglia invariably are included as an integrating part in these tumours, definite nerve cells have been ascertained previously only in one of the cases described by *Rocher & Anglade*. *Berblinger* points out that certain cells in his case

resembled nerve cells, but that the large nucleoli typical of these cells were lacking, as was also the tigroid substance which, however, does not necessarily occur in all the ganglion cells of the nervous system. In the case reported by *Guthrie & Dott*, strikingly large cells with coarse nucleoli were observed, which nevertheless were regarded as hypertrophic glia cells. It seems less surprising that medullary sheaths do not form part of these tumours, since the cerebral fibres begin to myelinize only at the end of the seventh embryonic month, i.e., at a stage when the separation of the glioma rudiment has undoubtedly already taken place. As regards the present case, apart from neuroglia of an ordinary character, also peculiar glial cells are to be seen, of the same kind as observed previously by several other investigators (e.g., *Siessenguth, Berblinger, Eigler; Bratton & Robinson*). However, in addition, typical ganglion cells have also been ascertained. Some nerve cells have had an appearance differing from the normal, but the occurrence of intracellular neurofibrils in these instances indicates that nerve cells actually are concerned here too. Their structure, which has changed in a regressive direction, may possibly be due to the inactivity of the isolated nerve cells.

A few authors (*Schmidt; Rocher & Anglade*) have ascertained in the so-called nasal gliomas a marginal connective tissue delimitation of the tumour from the skin, stating this connective tissue to be pial tissue, particularly in view of the fact that the connective tissue has descended into the tumour in broad streaks, imitating, as it were, conditions in the cerebral pia. Whether this really concerns connective tissue of meningeal origin seems uncertain. At any rate, it cannot be denied that the subcutaneous connective tissue under pressure from the tumour may assume this appearance. In certain instances, the connective tissue in the tumour has shown a general tendency to proliferation, justifying the diagnosis of fibroglioma (*Rocher & Anglade*).

Thus, from what has been said here, it is evident that the essential proof of the so-called nasal gliomas being severed cerebral elements, viz., the presence of nerve cells is lacking in the majority of the cases in the literature. In this connection, the question arises to what extent an encephalocele — in the author's opinion a phenomenon related to the nasal gliomas — discloses the structural peculiarity of the meninges and of the cerebral parenchyma. An interesting fact propounded among others by *Berger*, in his study of the pathology of the encephalocele, then emerges, namely, that certain encephaloceles are not »miniature brains« surrounded by unchanged meninges. Both the nerve cells and the meninges may have changed to such an extent as to motivate the conception of the encephalocele as a neoplasm, for which the term encephaloma has been suggested (*Berger*). The reason for this strange transformation is hard to decide, but it is probably, to some degree, due to the potential capacity of the tissue elements.

that have been severed at the embryonic stage to proliferate separately in abnormal quantities while lacking the physiologic formative stimulation.

The adequacy of the term glioma for these formations is now questioned. As emphasized by *Guthrie & Dott*, they should not be called genuine tumours, since the glia cells fail to disclose the embryonic characters justifying a diagnosis of neoplasm. In accordance with my own conception of the genesis of the so-called nasal gliomas, I would suggest the term *encephalochoristoma nasofrontalis*.

Summary.

In connection with an account of a case of so-called nasal glioma observed by the author in a child of two months, a survey is presented of cases of such tumours reported in the literature. From a histological point of view, the author's case conforms, broadly speaking, to earlier descriptions of these tumours. However, the occurrence of nerve cells is noteworthy, particularly in view of the fact that they have only been ascertained in one previously published case. As regards the genesis of the so-called nasal gliomas, the author shares the opinion that they constitute severances from the brain at an early embryonic stage, suggesting accordingly the term *encephalochoristoma nasofrontalis* for these formations.

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STUDIES ON BACTEROIDES¹⁾

I. INTRODUCTION

By Th. Thjøtta and Jon Jonsen.

(Received for publication April 28th., 1948.)

We have for some time been engaged in a study of the Gram negative anaerobic rods called *Bacteroides* by Bergey et al.¹⁾ During this work it has been obvious that few groups of microbes present such a confusing aspect as these microbes. By Bergey et al. they have been assembled in Fam. *Bacteriaceae*, Gen. VIII under the said name. In this genus we find 23 species. An appendix I of 6 genera named by A. R. Prévot²⁾ as RISTELLA, CAPSULARIS, ZUBERELLA, SPHEROPHORUS, SPHEROCILLUS and a species innominata contains 28 species. An appendix II is given containing a number (23) of bacterial species found in the literature but so poorly defined that their position is quite obscure. This gives as a total 74 different species, among which a Gram negative anaerobic non sporulating rod possibly might be enlisted, if the characteristics of the different species were so clearly stated that any bacteriologist working with adequate means should be able to place an organism answering to the main characteristics of the group.

The aim of all researchers in the field should naturally be that of giving well defined and clear descriptions of their microbes of so stable characters that other investigators could work out the characters of their own strains along the same lines and find those characters in their own strains. If, however, the description more or less is founded upon more personal impressions this description lacks value as a means of distinct classification, and other researchers will be handicapped in their endeavor to classify a strain found in pathological material. Any bacteriologist may have had the opportunity to find

¹⁾ This work has been made possible by a grant from Nyegaard & Co. (Nycø), for which we are very thankful.

for himself, that he is unable to classify such a strain, a fact that shows that the present classification does not rest on a so well founded base as that of the Salmonelleae or the Streptococci.

The classification of Bacteroides by Bergey et al. is mainly based upon the studies by A. H. Eggerth and B. H. Gagnon³⁾ in 1933 and those of A. R. Prévot et al. in 1937. The latter ones have for the greater part tried to classify organisms formerly described in literature, while the former ones have studied specimens of stools and described 18 different species found in these studies. From 65 stool-examinations 118 strains were isolated, but many of these were found only once or twice. It is reasonable to conclude that the examination of many more stools would have disclosed still more strains hitherto not described. Thus A. Distaso in 1911⁴⁾ and 1912⁵⁾ described 9 species from human stool while Eggerth and Gagnon only found 2 of these species in their examinations. Likewise H. Tissier⁶⁾ in 1908 described 3 strains from the same source, and none of these were found by Eggerth and Gagnon.

On going through the literature on this point it is remarkable that most of these Bacteroids described by the authors mentioned are not described by other researchers. This fact leads us to conclude that the amount of Bacteroids must either be very great or the classification must be of minor value to bacteriologists in their endeavor to find suitable lines in the chaos of this material.

Before we shall go into our own material we shall give a short resumé of those species of Bacteroides of interest for the human pathology and as such recognized by Bergey et al. We shall first mention a group of Bacteroids described by many researchers and so well defined that their characters can be recognized by other researchers.

B. fragilis.

This microbe has been found in the natural cavities of man and in different pathological conditions, such as appendicitis, periurethral abscesses, gangrena of the lungs, infections of the urinary tract, and in abscesses of the liver. The microbe is a small, Gram negative rod, growing slowly and sparsely in broth with clouding and a sparse and fragile sediment. It does not produce gas, indole or H_2S , but it acidifies a number of carbohydrates, but does not attack milk although some strains splits lactose.

B. funduliformis.

This microbe was first described by A. Veillon and A. Zuber⁷⁾ in 1894 and by J. Halle⁸⁾ (1898) from the female genitales. It has been found in many infectious conditions in man, such as otitis, gangrena of the lungs, infections of the urinary tract, parametric abscesses,

pyemia with metastases of the liver and in many other pathological conditions. The local infections caused by this organism are always characterized by fetid, gangrenous and necrotic inflammations. *B. funduliformis* is characterized as a polymorphous, non motile microbe consisting of rods of different length bearing many spheroid, oval or irregular bodies, that also may appear as free constituents of the culture. The microbe will grow in broth with a more or less dense sediment and it forms a fetid smelling gas. Gelatine is not liquified, indole is only produced by old strains and the formation of H_2S is slight. It grows without hemolysis on the blood agar plate, but produces hemolysin in culture. The fermentation of sugars is quite various.

B. serpens.

This microbe was found in the same year (1898) by Veillon and Zuber⁹⁾ in appendicitis, by Rist¹⁰⁾ in mastoiditis and by L. Guillemot¹⁰⁾ in gangrena of the lungs. Since then it has been described by several authors. It occurs single or in short chains and grows with clouding of the medium and forms later on a sediment in broth. It does not produce H_2S , but liquifies gelatine slowly and forms acid in different sugars. The microbe is motile by the means of slow undulations.

B. melaninogenicus

was first isolated by W. W. Oliver and W. B. Wherry¹¹⁾ in 1921 and later on described by several authors. It is found as a saprophyte on the mucous membranes in man and in different pathological processes such as sepsis, abscesses of the salivary glands and gangrena of the lungs. The main characteristics of this microbe is that it is a hemophilic and hemolytic organism, producing a coal black pigment on blood agar plates. Growth is very poor in the absence of body fluids. On prolonged growth the black pigment may be absorbed and disappears.

Together with these fairly well defined species of *Bacteroides* several species have been described that show a great similarity to *B. funduliformis*. In listing these species we shall only mention those characters that according to Bergey et al. distinguish them from that organism.

B. necroticus is only isolated once by R. Natipelle¹²⁾ from appendicitis. It is distinguished from *B. funduliformis* by a more or less viscid culture (glucose broth) and by a large production of globoid bodies in culture.

B. floccosus was isolated by Couremont and Cade¹³⁾ (1900) from a case of septicopyemia and by Franklin¹⁴⁾ in 1933 from sepsis. It distinguishes itself from *B. funduliformis* in hemolysing blood agar plates, in not producing gas and in no action upon sugars.

B. mortiferus isolated once by N. M. Harris¹⁵) (1901) from abscessus of the liver and distinguished from *B. funduliformis* in production of H_2S and indole and in the ability of peptonizing milk with production of acid without coagulation and in growing only in media containing body fluids.

B. freundii was isolated by F. Freund¹⁶) (1922) from meningitis. It is distinguished from *B. funduliformis* in the production of indole and in not growing without body fluids on the first isolation.

B. bullosus was found once only by A. Distaso⁵) (1912), and is quite similar to *B. funduliformis* with the only exception that it is described as motile. It is of interest to mention that several of Distaso's microbes are described as motile, while other authors have found them not motile. It may be that Distaso has not differentiated clearly enough between real motility and Browns motility.

It will be observed that these last mentioned »species« are enlisted as species sui generis on the presumption that:

1. *B. funduliformis* does not produce spheroid bodies in cultures.
2. *B. funduliformis* does not hemolyse blood agar plates.
3. *B. funduliformis* does always produce acid and gas, but not H_2S and indole in young cultures.
4. *B. funduliformis* does not peptonize milk.
5. *B. funduliformis* will never need body fluids for growth in the primary isolation.

It seems obvious that one of these characters alone must be a very slight basis for the statement of new species, when all other main characters are the same. We shall have the opportunity later to show that several of the distinctions made in this way are imaginary.

Another group of Bacteroides is that of non pathogens of rather doubtful characterization and found by only one author. They fall presumable into different species, but the characters thought to be distinct for each species are of very doubtful value. Thus production of indole and of H_2S is claimed of so highly specific importance that differentiation of species may be founded upon these single characters. Variation of the acid production in sugars is likewise used freely in taxonomic work. Some of these microbes are described as acidifiers of milk without coagulation, and some are claimed to produce acid in lactose, glucose and galactose, while they will grow in milk without causing any alteration of this medium.

We shall mention some of these microbes quite shortly:

B. vesus, *B. uncatius*, *B. exiguus*, *B. tumidus*, *B. convexus*, *B. ovatus*, *B. gulosus*, *B. siccus* all isolated by Eggerth and Gagnon from human faeces, in the same series of examinations (1933). The main charac-

ters of these species were their homogeneous growth in broth and the liquefaction of gelatine. Further the following species showed homogeneous growth in broth but failed to liquefy gelatine: *B. various*, *B. insolitus*, *B. inaequalis*, *B. incommunis*, *B. thetaiomicon* (named after the greek letters theta, iota and micro). *B. thetaiomicon* is also found by Distaso, but described as a motile rod. To the last group are related two species which are said to show a varying liquefaction of gelatine, namely *B. uniformis* and *B. distasonis*. Of the former species 6 strains showed liquefaction, while 2 did not. Of the latter 4 strains did liquefy, while 16 did not, 16 strains showed coagulation in milk, and 4 did not, 2 strains hemolyzed bloodagar, while 16 were negative on bloodagar.

It seems justified to criticize the naming of species on characters of so doubtful taxonomic value as done here. It is not to be wondered at that the chaos of species must be overwhelming, when the basis for taxonomy is taken so easily as here. It will be noted that in some genera the difference is very slight between species, while strains of opposite characters are grouped in the same species.

The following group of 5 species of *Bacteroides* seems to be somewhat better characterized than many of the other groups. They are partly found only once and partly several times, mostly from human stool.

B. coagulans and *B. vulgatus*, both isolated by Eggerth and Gagnon. The former does not attack sugars, but produces gas in peptone water, indole in broth and liquefies gelatine, while the latter produces acid and gas in sugars, but produces no gas in peptone water. The other characters are very much alike.

The following 3 species produce mucoid colonies and are capsulated. They are *B. variabilis*, *B. mucosus* and *B. zooglyphiformans*. The second one is characterized by the formation of fetid gas, while the last one does not produce any gas, while both these species craved body fluids for their isolation.

Last we shall mention quite shortly a group of *Bacteroides* mostly classified by Prévot. It is a common characteristic also for this group, that the species often are founded on the isolation of one single strain, two strains or sometimes a few strains. The differentiation between species are based upon characters earlier mentioned in this paper and also upon slight differences in the morphology. It seems quite interesting that many of these species seem to occur very seldom or what may be suspected show a high degree of variability and thus lead the investigator to consider the same microbe as belonging to different species under different conditions.

These species are as follows:

Ristella haloseptica, *Ristella putriednis*, *Ristella terebrans*, *Ristella*

trichoides, *Ristella furcosus*, *R. capillosus*, *R. cylindroides*, *R. perfoetens*, *Zuberella rhinitidis*, *Z. preacuta*.

These species have been obtained from various purulent inflammations, such as osteomyelitis, appendicitis, cholecystitis, rhinitis and from human intestines.

To summary this survey one may say that the systeme of *Bacteroides* at present is a deplorable turmoil of names placed on what is called species of a very doubtfull reality. The authors obviously have been tempted to regard one single strain as a special species, when they on isolation find that it differs in some, often quite slight degree from other species. And we are justified in believing that the investigation of such strains often has been carried out with technique different on essential points, and thus giving different results in the hands of different investigators. We may mention such a delicate test as the test for H_2S . The technique here is of a paramount importance, as a poor technique may give a negative result where a better one gives a positive one for the same strain. Another fact may be mentioned. Some authors may use a homogeneous growth in broth as opposed to a flocculate one as a means of differentiation into species although it is well known that a rough strain of a microbe will grow flocculate in broth, while the smooth strain may grow uniformly cloudy. Still other authors differentiate strains into species according to slight differences in morphology such as the individual elements being longer or shorter rods.

It is obvious from these short remarks, that one must be justified in doubting the validity of a great deal of the classification of the Gram negative, non sporeforming anaerobic rods and in wishing for a greater interest in the investigation of the group along modern technical lines with the aim of a more simple and clear classification, where the species may be based on the main characters, while minor differences of doubtful importance are neglected.

We have long felt that the differentiation between *B. funduliformis* and *Necrobacterium* (*Actinomyces necrophorus*) may be illusory and will treat this question in our next paper of this series.

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STUDIES ON BACTEROIDES

II. *B. FUNDULIFORMIS* AND ITS RELATION TO *NECROBACTERIUM NECROPHORUS* (*ACTINOMYCES NECROPHORUS*)

By *Jon Jonsen and Th. Thjøtta.*

(Received for publication April 26th 1948.)

The characters and the systematic position of *Necrobacterium necrophorus* (*Actinomyces necrophorus* of Bergey et al.) has been dealt with in this journal by *Th. Thjøtta* and *O. Lahelle*¹⁾ and in the large monography by *Lahelle*.²⁾ We shall refer to those papers and shall not take up the description of *Necrobacterium* in detail, but only so far as it is necessary for comparison with *B. funduliformis*.

Since we began our studies on *Bacteroides* and especially on *Necrobacterium* we have held it very likely that this microbe and *B. funduliformis* in reality are identical, and if not identical so closely related to each other, that it seems impossible to make a clear distinction between them using morphological and biochemical methods alone. We have therefore taken the trouble of doing a close examination of *B. funduliformis* morphologically and biochemically and have compared the characters so found with those found by us and by *Lahelle* in a material of 20 strains of *Necrobacterium*, and in this study we have used the same methods as those used in the said studies of *Necrobacterium*.

As is well known both bacteria in question consist of Gram negative rods of different length or non branching threads together with round or ovoid bodies, the latter both occurring quite free or in the rods as irregular swellings. Also biochemically the two species appear to be very much similar in characters such as production of gas in carbohydrates, hemolysis in cultures, H_2S production and others. The pathogenicity is also mostly the same, although *B. funduliformis* is found in infections in man and *Necrobacterium* is found especially in animals. This fact may be explained simply as a different naming of the same microbe when found by human and by veterinary bacteriologists,

moniliformis, although in this case with a larger occurrence of the chainlike elements.

Cultural characters.

B. funduliformis grows well on solide media appropriate for the growth. The colonies vary in appearance both according to their age and (although to a lesser degree) to the composition of the medium. They show a smooth appearance transparent and greywhite of color as quite young colonies. But in the lapse of 3—5 days of growth, when they reach a diameter of 3—4 mm., many of them will show a rough appearance, although some will remain small and still keep their smooth growth. In the following days of growth, as the colonies grow old, the edge becomes irregular, the surface loses its shiny appearance and becomes matt, the colony as a whole becomes flatter with a dark centre. At last the centre sinks into the colony, while the edge is slowly raised and becomes mucoid. The consistency is still pastelike rather than slimy, and the color is now dirty yellowish green. Round the colonies a broad metal glistening halo is seen both in plain media and on blood agar plates.

The hemolysis of blood agar is somewhat caprizious. While the rule is that both our strains show a complete and strong hemolysis on sufficient incubation, they may now and then give non hemolytic colonies both of them or only one strain at a time. This phenomenon could not be explained as a result of the medium as one strain might give a good hemolysis, while the other did not hemolyse on the same plate, and this condition might be vice versa in the next experiment. However, the hemolytic property is distinct and can be seen on human blood as well as on animal blood (sheep-, horse- and cow-blood).

Lahelle in his description of the colonies of *Necrobacterium* mentions S, R and intermediate colonies. He also describes mucoid colonies, and he has also seen the sunken centre in his mucoid colonies just like those described by us in *B. funduliformis*. The other colonial types described by Lahelle in all respects resemble those in our strains of *B. funduliformis*, and we will especially mention the peculiar metallic shine produced by both *B. funduliformis* and by *Necrobacterium*. The consistency and the hemolysis are also quite alike.

It is naturally obvious that many different species of bacteria may show characters exactly like those of other microbes, without being related to each other. But in the case of *B. funduliformis* and *Necrobacterium* the characters can all of them be found exactly similar in both microbes both as belongs to the morphological picture as to that of the behaviour of the colonies.

In a high agar column grey lenslike colonies are formed with more or less gas around themselves. The growth appeared to a distinct line underneath the surface of the column. In gelatine the strains also

grew quite well with a flocculent sediment in the bottom of the culture tube. No liquefaction appeared.

The growth in fluid media was slightly different in our two strains. *B. funduliformis* 267 always grew with a flocculent sediment with a clear fluid above the sediment. *B. funduliformis* C.H., however, in young cultures and especially in rich media, showed a homogeneous clouding of the medium. The old cultures, however, successively developed a flocculent sediment like that of the other strain.

In his description of the growth of *Necrobacterium* in fluid media Lahelle mentions that most of his strains grew with clouding and sediment, while 5 strains showed flocculent sediment and very slight clouding only.

The fermentation of sugars was tested in 2 per cent peptone water added 1 per cent of glucose, levulose, lactose, mannitol and saccharose. The two first sugars were fermented with the production of acid and gas after 4—5 days of growth, while the cultures in the three last ones did not show any sign of fermentation after a period of growth of 14 days. The same fermentation was found by Lahelle in his study of *Necrobacterium* with the only exception that one of his strains did ferment saccharose, while all the others did not.

Our two strains produced a strong reaction of indole (Ehrlich's indole reaction) when grown in ordinary broth. H_2S was also produced in gelatine with ferri chloride, while this reaction was more difficult to define in media added lead acetate. Growth of the strains in cysteine glucose broth cultures added small pieces of iron gave an intensely black coloration which was taken as a sign of the production of H_2S .

Lahelle in his material of *Necrobacterium* found that all his strains were indole and H_2S producers.

Weinberg, Nativelle and Prévot (3) find no production of indole in *B. funduliformis*, while Kisskalt (4) mentions that the microbe produces indole in old strains. Likewise the former authors claim that no production of H_2S takes place in *B. funduliformis*, while Tissier (5) has found a small amount of H_2S in his cultures of *B. funduliformis*. As already mentioned the two strains examined by us are sent us by Prévot, and must consequently answer to the characters claimed by Prévot et al. for *B. funduliformis*. When these two strains in our hands produce both indole and H_2S , we can only conclude that these characters of the strains have not been disclosed by the method used by Prévot.

Growth Factors in the Cultivation of B. funduliformis.

All cultural experiments with *B. funduliformis* show that this microbe can grow upon ordinary media. Distinct experiments as to the necessity of different growth factors have not, however, been made, although it is obvious that the growth becomes more abundant on the

addition to the ordinary media of different compounds. We have therefore tested the effect of such additional factors as glucose, cysteine, ascites, potato and potato extract on the yields of growth in *B. funduliformis*.

The technique of these experiments has been as follows. The basal medium has been a very poor one, namely 1 percent peptone water. The inoculum has been the centrifugal sediment from a 48 hours culture in glucose-cysteine broth, after washing of this sediment in the centrifuge and resuspension in normal saline, to a standard of 1000 mill. microbes per ml. A single drop of this suspension has then been added to the different media. The tubes containing the basal medium were heated in a boiling water bath before inoculation and the incubation took place in an anaerobic metall jar evacuated in an air pump and washed thrice with hydrogen before incubation.

A preliminary test with 1 and 2 percent of peptone water was carried out before the main tests in order to state the faculty of growth in this medium. The result was as stated in table I.

Table I.

Medium	Days of cultivation	Amount inoculum in drops of suspension					
		1	2	4	6	8	10 drops
1 percent p. w.	2	—	—	—	—	—	++
	4	—	—	—	—	+	++
	10	—	—	—	—	+	++
2 percent p. w.	2	—	—	—	—	—	++
	4	—	—	—	+	++	++
	10	—	—	—	++	++	++

Table I shows that *B. funduliformis* is able to grow in 1 or 2 percent of peptone water, if the inoculum is high, but not if the inoculum is very small. The same observation was made by Lahelle with his strains of *Necrobacterium*. These were unable to grow in these solutions of peptone immediately after isolation from the animal body, but when old strains were tested they did grow if the inoculum was high. As our strains of *B. funduliformis* are old strains the comparison with *Necrobacterium* consequently is complete.

In Table II our results of cultivation of *B. funduliformis* in different concentrations of glucose added to the basal medium (1 percent of p.w.) are shown.

Table II.

Strain	Days of cul- tivation	Peptone water with glucose per cent													
		0.05	0.1	0.2	0.40	0.8	1	1.5	2	3	4	5	7	8	9
B. funduli formis C. H.	1	—	—	—	—	+	+	+	++	+	—	—	—	—	
	2	—	—	—	++	++	++	++	++	++	++	++	—	—	
	4	—	—	+	++	++	++	++	++	++	++	++	—	—	
	10	—	—	+	++	++	++	++	++	++	++	++	+	—	
B. funduli- formis 267	1	—	—	+	++	++	++	++	++	++	+	—	—	—	
	2	—	—	++	++	++	++	++	++	++	++	++	++	—	
	4	—	—	++	++	++	++	++	++	++	++	++	++	—	
	10	—	++	++	++	++	++	++	++	++	++	++	++	—	

Table II shows that glucose has a distinct promoting effect on the growth ability of *B. funduliformis* even in so low concentrations as 0.4 and 0.2 per cent. The intervals of the most pronounced effect, however, lies between 0.8 and 4 per cent of glucose. In the higher concentrations the growth is poorer and above 8 per cent no growth takes place.

To ensure growth of *B. funduliformis* in several generations 0.8 per cent of glucose in 1 per cent of peptone water is the smallest quantity of this chemical that can be used. 1 per cent has therefore been used in our study to this aim just as Lahelle in his study of *Necrobacterium* found that just this addition of 1 per cent of glucose was best suited for this use.

The growth promoting faculty of cysteine hydrochloride was tested in the same manner as stated for glucose. Table III shows these experiments.

Table III.

Strain	Days of cultivation	Peptone water with cysteine hydrochloride in per cent												
		0.004	0.006	0.008	0.01	0.012	0.014	0.016	0.018	0.02	0.04	0.06	0.08	0.
B. f. C. H.	1	—	—	—	—	+	+	+	+	++	+	—	—	—
	2	—	—	—	—	++	++	++	++	++	++	++	—	—
	4	—	—	—	+	++	++	++	++	++	++	++	—	—
B. f. 267	1	—	—	+	++	++	++	++	++	++	++	+	—	—
	2	—	++	++	++	++	++	++	++	++	++	++	—	—
	4	—	++	++	++	++	++	++	++	++	++	++	—	—

Table III shows that cysteine hydrochloride favors the growth of *B. funduliformis* in quantities of 0.01 per cent up to 0.08 where an inhibiting effect sets in. *B. f.* 267 can start its growth on a somewhat lower per cent of the factor than *B. f.* C.H., although the inhibition starts on the same point. Also this chemical permits a continuous

growth of both strains for several generations when a concentration of 0.012 to 0.06 per cent is added to peptone water.

In his examination of *Necrobacterium* Lahelle found that an addition of 0.03 per cent of cysteine hydrochloride to 2 per cent of peptone water gave an optimal growth, but the chemical favored the growth in concentrations ranging from 0.01 up to 0.05 per cent.

The growth favoring effect of ascitic fluid has been examined in the same manner as that of the two former factors. Table IV shows the results of these tests.

Table IV.

Strain	Days of cultivation	Peptone water with ascitic fluid in per cent										
		1	2	4	5	7	10	15	20	30	40	50
B. f. C. H.	1	—	—	—	—	—	+	++	++	++	++	++
	2	—	—	—	—	—	++	++	++	++	++	++
	3	—	—	—	—	+	++	++	++	++	++	++
	4	—	—	—	+	++	++	++	++	++	++	++
B. f. 267	1	—	—	+	+	+	+	++	++	++	++	++
	2	—	—	+	+	+	++	++	++	++	++	++
	3	—	—	++	++	++	++	++	++	++	++	++
	4	—	—	++	++	++	++	++	++	++	++	++

Table IV shows that an addition of 15 per cent of ascitic fluid or more promotes an optimal growth of *B. funduliformis* and also that strain 267 here as in the former tests show a greater willingness of growth. Also for the continuous growth in several generations the concentration of 15—20 per cent of ascitic fluid is well suited.

Lahelle in his study of *Necrobacterium* (and in his recently isolated strains) found that 20 per cent up to 40 per cent gave a good growth.

The effect upon growth of whole blood, red cells, serum and plasma was next examined. Table V shows the results of these tests.

Table V.

Strain	Days of cultivation	0.05 per cent				0.1 per cent				0.5 per cent			
		Blood	serum	plasma	red cells	Blood	serum	plasma	red cells	Blood	serum	plasma	red cells
B. f. C. H.	1	—	—	—	—	—	—	—	—	++	++	++	+
	2	—	—	—	—	+	—	—	—	++	++	++	+
	3	+	—	—	—	++	++	++	++	++	++	++	+
	4	+	—	—	—	++	++	++	++	++	++	++	+
B. f. 267	1	+	—	—	+	++	++	—	++	++	++	++	+
	2	+	—	—	+	++	++	+	++	++	++	++	+
	3	+	—	—	+	++	++	++	++	++	++	++	+
	4	+	—	—	+	++	++	++	++	++	++	++	+

Table V shows that whole blood has a higher value as a growth promoting factor than serum, plasma and red cells. There is perhaps a slightly better effect of red cells than of serum and plasma, but those latter ones show nearly the same effect. Quite clearly the quantities of the different blood fractions are of distinct importance. The least quantity showing a good effect is 0.5 per cent of the different fractions. Lahelle in his studies showed that whole blood also in *Necrobacterium* gave the best results, and that quantities from 0.1 up onwards could start and sustain growth in close agreement with our results with *B. funduliformis*.

A convenient growth promotor for various microbes is sterile pieces of potato. It will be remembered that potato contains both the X and the V factor of *Thjötta* and *Avery*, and that anaerobes may grow well in broth added small pieces of potato. This medium has been extensively used both in Lahelles studies of *Necrobacterium* and in our studies of *B. funduliformis* and found well suited for the cultivation of both microbes. Lahelle found that pieces answering to a dimension of 10 mm³ gave good growth just as we have found that very small pieces can sustain the growth. Also sterile extracts of potatoes can be used. We found an optimal growth in quantities of 5—20 per cent of extract, but also 0.5 per cent could be used. Lahelle found a promoting effect of such extracts in quantities of 2 per cent, but did not try smaller amounts.

Metallic iron is known to stimulate the growth of anaerobes and was as such used both by Lahelle and by us. We used a nail of 3 inches length in 10 ml. cysteine-glucose broth. This medium with the nail was boiled for 20 minutes before inoculation with material from a brain heart agar culture. The influence of the iron was that inocula too small to give any growth in the medium without iron gave a good growth when iron was present. More prominent than this growth promoting action is the intense blackening that takes place in the iron medium and makes the culture quite intransparent. Together with this change of color a strong development of gas with a very intense fetid odour took place. We have found that this phenomenon is a sign of development of H₂S by the microbe.

The temperature of choice for the cultivation of *B. funduliformis* is 37° C., but growth can be obtained between temperatures from 25 to 42° C. Even lower and higher temperatures such as 23 and 44° C. might give growth, but at these temperatures growth was irregular and at the highest temperatures gas production was very sparse or absent. The highest temperature that allowed growth of *B. funduliformis* is also that which Lahelle found to give growth in his study of *Necrobacterium*, namely 44° C. The minimum temperature permitting growth was 22° C. in *Necrobacterium* and 23° in *B. funduliformis*.

The intervalls of pH where the two microbes gave their best growth are practically identical, *Necrobacterium* growing most abundantly

between pH 7.5 and 7.8, while *B. funduliformis* had a growth maximum between pH 7.3 and 7.78. Growth was, however, present between 5.80 and 8.27 in *B. funduliformis* and between 5.69 and 8.37 in *Necrobacterium*.

Thus it seems obvious that both temperature and pH conditions show practically the same influence upon the growth of *B. funduliformis* and *Necrobacterium*.

B. funduliformis is a strict anaerobe demanding very well established anaerobic conditions. Growth in high agar reaches up to about 2 cm below the surface and up to 0.5 cm in semisolid agar. On inoculation on solid media growth was quite good in anaerobic jar provided the inoculation was made from the same kind of medium and fairly abundantly. Media that had been kept in open air did not give good growth as the rule is with other anaerobic microbes. Our results as to the sensibility of *B. funduliformis* to aerobic conditions are completely parallel to those obtained by Lahelle in *Necrobacterium*.

The hemolytic action of *B. funduliformis* and of *Necrobacterium* is of value in diagnostic examinations of the two microbes. This effect is seen on blood agar and can be examined more in detail in fluid media, where a titration of the hemolysin can be done and a comparison carried out as to the influence of the hemolysin upon different kinds of blood.

The study of the hemolytic exotoxin has been carried out in broth, flasks of 50 ml. being inoculated with 1 ml. of a brain heart agar culture after thorough heating to drive off all air contained in the medium. The flasks were incubated in an anaerobic jar for 20 hours, when the culture was centrifugalized and part of it was filtered through Berkefeld candles N. The two fractions, one only centrifugalized and the other also filtered, were then titrated from 0.5 downwards and added 0.5 ml. of washed red cells of different bloods in a concentration of 5 000 mill. per ml. The experiments were carried out in a water bath at 37° C. Table VI shows the results obtained with *B. funduliformis* C.H.

The same experiments were carried out with hemolysin produced by *B. funduliformis* 267 with very similar results. It is obvious that the hemolysin is to a certain degree absorbed in the filters when passed through a Berkefeld candle just as the case is with other hemolytic enzymes. It is also clear that all the different kind of blood examined, i.e. from sheep, horse, rabbit and ox is hemolysed almost to the same degree, although there is a slightly higher resistance to the hemolysis in the blood from rabbit and horse than in that from sheep and ox.

Human blood has also been examined as to the sensitivity towards the hemolytic action of *B. funduliformis*. Table VII shows an experiment carried out in this connection. The hemolysin was tested in unfiltered condition.

Table VI.

	Red cells from	Time of incubation hours	Doses of hemolysin						
			0.5	0.4	0.3	0.2	0.1	0.05	ml. control
Unfiltered broth.	Sheep	1	100	100	100	100	0	0	0
		4	100	100	100	100	100	100	0
	Horse	1	100	100	100	75	0	0	0
		4	100	100	100	100	100	100	0
	Rabbit	1	100	100	50	25	0	0	0
		4	100	100	100	100	100	100	0
	Ox	1	100	100	100	25	0	0	0
		4	100	100	100	100	25	25	0
Filtered broth.	Sheep	1	25	25	25	0	0	0	0
		4	25	25	25	0	0	0	0
	Horse	1	50	25	25	0	0	0	0
		4	100	100	75	75	25	0	0
	Rabbit	1	75	50	25	25	0	0	0
		4	100	100	100	75	25	0	0
	Ox	1	25	25	0	0	0	0	0
		4	100	100	100	25	25	25	0

100 — 100 per cent hemolysis, 0 — no hemolysis, 75 50 25 resp. partial hemolysis.

Table VII.

Strain	Blood from	Doses of hemolysin						
		0.5	0.4	0.3	0.2	0.1	0.05	ml. control
C. H.	Male H. H.	100	100	100	100	25	0	0
	Male J. J.	100	75	75	0	0	0	0
	Woman T. J.	100	100	100	100	75	0	0
	Woman J. J.	100	100	50	50	50	50	0
	Sheep	100	100	100	100	100	50	0
267	Male H. H.	100	100	100	0	0	0	0
	Male J. J.	100	0	0	0	0	0	0
	Woman T. J.	100	100	75	0	0	0	0
	Woman J. J.	50	25	0	0	0	0	0
	Sheep	100	100	25	0	0	0	0

Table VII shows that both our strains of *B. funduliformis* produce an hemolytic factor for human blood and that the effect of that produced by strain 267 is slightly weaker than that of strain C.H. It also shows that the sensitivity of blood from different individuals is not exactly the same, but may vary to some degree. Even the blood from the same individual may show different hemolytic titres from time to time. This fact may probably be explained as the result of technical differences such as the variation in growth capacity of the strain, small differences in the medium, slight differences in the collecting and keeping of the blood etcetera.

Lahelle also finds that his strains of *Neerobaeterium* produce a hemolytic factor as showed both in the hemolysis on blood agar plates and in test tube experiments. There are some minor differences between those features of hemolysis claimed by Lahelle for his strains and the same in our experiments. The most important of these differences is that Lahelle finds that blood agar produced with blood from horse, goat or sheep only showed a green coloration instead of a hemolysis, while blood from man, rabbit or ox gave a distinct hemolysis. We do not attach much importance to this discrepancy between the results of Lahelle and our own results. The fact remains that both these organisms, *B. funduliformis* and *Necrobacterium* produce a hemolytic factor active as well in solid cultures as in fluid media and that this factor is efficient as well against human blood as against the blood from several species of animals. The action of this hemolytic factor is, however, not very strong, a fact that most likely explains why both these microbes partly are named as hemolyzers partly as non hemolyzers. And this is probably the reason why the hemolytic power only rarely is taken into consideration when the diagnosis is made on bacterial strains otherwise answering to the main characters of either *Neerobaeterium* or *B. funduliformis*.

Discussion.

This paper relates the results of a morphological, biochemical and a cultural examination of *B. funduliformis* as presented by two strains obtained from Prévot and named by him *Spherophorus funduliformis* C.H. and 267. The characters so found by us are compared to those found by O. Lahelle in our institute in his study of a microbe very much similar to *B. funduliformis*, as we prefer to name the first one. It will be remembered that Bergey et al. *) have placed both these microbes as different species in the same genus, namely the *Bacteroides*. The microbe studied very extensively by Lahelle is that one called by Bergey et al. *Actinomyces necrophorus*, and by us preferably called *Necrobacterium necrophorus* or only *Neerobaeterium* as the genetic name should be.

We have been interested in making a through comparison between:

*) Edition 5 (1939).

these two species of bacteria and will show below the characters and discuss these in comparison with each other.

According to Prevot the morphological picture of *B. funduliformis* is characterized by the occurrence of Gram negative rods or threads with or without spheroid bodies in the culture. This picture is so characteristic that if a sample of pus coming from human origin gives this picture in culture (in anaerobiosis) one may presume that the pus contains *B. funduliformis* (Prévot).

It is quite clear that the so-called spheroid bodies both in the threads and as free bodies are found in *B. funduliformis*. This is, however, also the case with several other microbial cultures, and quite especially with cultures of *Necrobacterium* as shown by Lahelle and many other writers. We have also found these same structures in *Haverhillia moniliformis* as mentioned already. The main point however, is that the morphological picture cannot give any distinction between *B. funduliformis* and *Necrobacterium*.

Lahelle has placed great importance upon the growth of his strains on a solide mediums surface, while Prevot et al. does not attach much importance to this phenomenon. However, Lahelle has found both S, R and intermediate colonies in his cultures and after 3 days of growth some times a mucoid transformation of the colonies, in that the centre of the colonies gradually sunk and the edge was raised and became mucoid. In our cultures of *B. funduliformis* this phenomenon was seen often while we kept our cultures under surveillance for a longer time than Lahelle. The other colonial aspect, however, was the same in our cultures as those in Lahelles material. The colonial types are accordingly unable to give a distinction between the two microbes.

The hemolytic action upon blood agar plates may be a good diagnostic distinction between *B. funduliformis* and other non hemolytic *Bacteroides*, but not between that microbe and *Necrobacterium*, since they are both hemolyzers.

The conditions of growth are the same for *B. funduliformis* and for *Necrobacterium*. They both give a very poor growth in peptone water dependant upon the quantity of inoculum. They are both and to the same degree influenced by different growth promoting factors, such as glucose, cysteine hydrochloride, ascitic fluid et cetera. Both are gas producers and produce H_2S , and indole. They both reduce nitrate and do not liquefy gelatine. The influence of temperatures and pH is the same in both species, likewise the sensitivity against oxygen and other smaller characters are closely the same. The action upon carbohydrates in our two strains of *B. funduliformis* is found in several of the *Necrobacterium* strains of Lahelle.

Prévot et al. put some importance upon the growth of *B. funduliformis* with a flocculate sediment in broth, while *Necrobacterium* presumably should grow with a homogeneous cloudy culture. This is however, not the rule. Lahelle has shown that some of his strains of

Necrobacterium did grow with a distinctly flocculate sediment. Besides, the two strains of *B. funduliformis* obtained from Prévot show a different growth in this connection. The one, C.H. grows with a cloudy culture in young condition while the other very often shows a flocculent sediment with a clear broth on top. The growth in fluid media cannot accordingly be of any use in the distinction between these two species, the more so because any microbe turning to the R side will more or less sediment.

We have consequently not found any means by which we could make a clear distinction between *B. funduliformis* and *Necrobacterium* in the morphological, biochemical or cultural aspect of the two species. The french authors maintain that the said characters are those upon which the distinction is made. We cannot agree in this conception, but assume that *B. funduliformis* and *Necrobacterium* is the same bacterial species, the first found hitherto in human beings, the latter in infections in animals. The pathological effect in both cases is the same, namely purulent inflammations with necroses. It remains, however, to make a serological comparison.

Conclusions.

1. Two strains of *B. funduliformis* have been examined in detail.
2. The morphological picture of these two strains are exactly the same as that shown by *Necrobacterium*, namely rods, threads, spheroid bodies occurring as well in the rods or threads as free in the cultures.
3. The biochemical characters of *B. funduliformis* and *Necrobacterium* found in this investigation are the same to the least detail.
4. The hemolytic powers in *B. funduliformis* and *Necrobacterium* are the same with a very slight discrepancy that may be explained on technical grounds.
5. All cultural characters such as ability of growth in different media and activation by different growth factors are identical.
6. On morphological, biochemical or cultural characters alone it is impossible to make a distinction between *B. funduliformis* and *Necrobacterium*.
7. It remains to find any possible distinction in serological experiments.

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Fig. 1.
B. fundulisformis 267.
 4 days growth on agar. (12 ×)

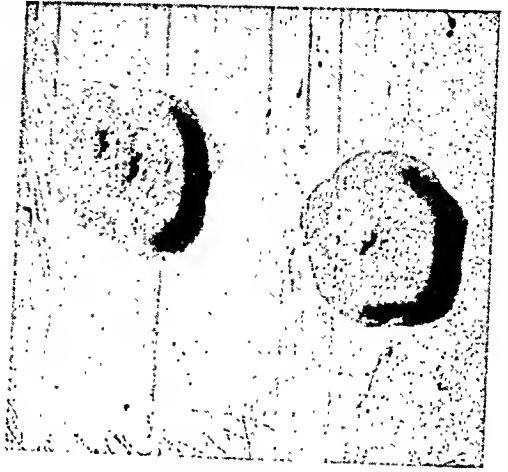


Fig. 2.
B. fundulisformis CH.
 4 days growth on agar. (12 ×)

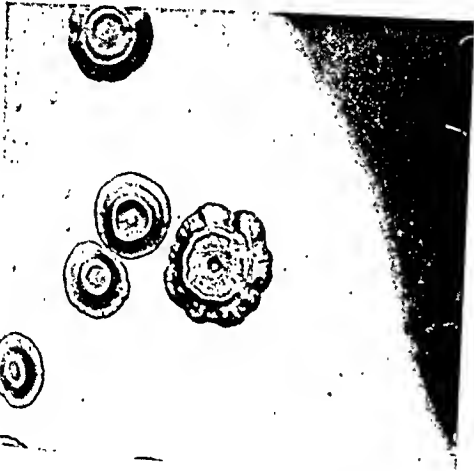


Fig. 3.
B. fundulisformis CH.
 Colonies on blood agar after 8 days
 growth. (8 ×)



Fig. 4.
B. fundulisformis 267.
 Broth after growth in 48
 hours. (1200 ×)

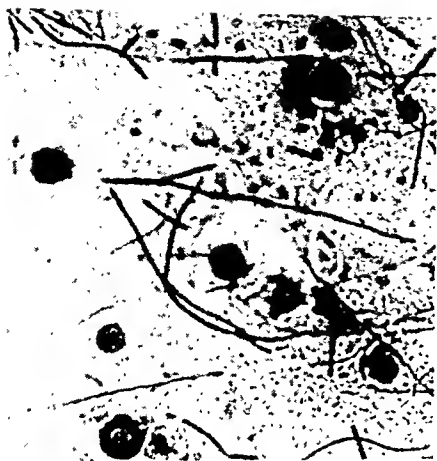


Fig. 5.
Necrobacterium ($\times 1200$).

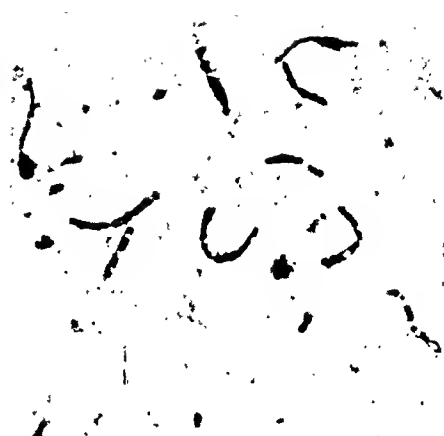


Fig. 6.
B. Funduliformis 267 ($\times 1200$).

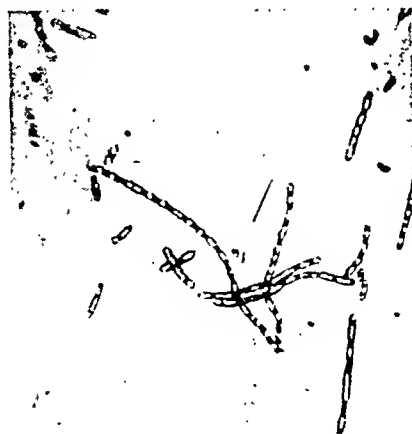


Fig. 7.
Necrobacterium ($\times 1200$).

ON THE SEROLOGY OF THE PROTEUS GROUP

By *Beate Perch.*

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In a previous paper of F. Kauffmann and Beate Perch »On the Occurrence of Proteus X Strains in Denmark«, the pattern for a Proteus antigenic schema was established describing the first 6 O groups out of 26 O groups known at that time.

The present paper is a continuation of that work and extends the antigenic table in both an elaborated and a simplified form. The former gives the results of exhaustive serologic diagnosis in regard to the O and H antigens, while the latter presents a Proteus antigenic schema suitable for practical use as recommended by Kauffmann and Perch (1). The results of the extended investigations will be stated only in brief, since they are intended for later publication as a monograph.

Material and Technical Methods.

The material was the same as that employed in the above-mentioned paper (1) and consisted of 540 freshly isolated strains of Proteus, together with a number of classical strains, derived from the following sources: feces (F) 447 cultures, urine (U) 47 cultures, pus (P) 41 cultures, autopsy (S) 4 cultures, heartblood of a mouse (M) 1 culture and 4 laboratory strains, X 19, XL, X 2 and X K from the National Collection of Type Cultures, Lister Institute, London, test strains for the first 3 O groups and 1 strain KP 21, identical with X 19 and formerly isolated here in Denmark by Kauffmann and Perch (2).

The techniques employed have on the whole followed the techniques used for Salmonella diagnosis, described by Kauffmann in »Die Bakteriologie der Salmonella-Gruppe«, 1941 (3).

To exclude the not infrequent occurrence of mixed cultures (i. e. the existence of 2 different types in the same culture) the original transplant was spread on agar plates containing 0.1 % phenol, in order to secure single colony isolation.

The determination of O and H antigens was made in tube agglutina-

tion with 20-hour broth cultures heated for 1 hour at 100° C, and with 6-hour formalinized broth cultures, respectively.

O immune sera were prepared by immunization of rabbits with 20-hour broth cultures heated at 100° C for 2½ hours, while OH immune sera were produced by inoculation of 6-hour formalinized broth cultures.

The determination of O antigens of O groups 1 to 30, inclusive, was made with O sera, whereas the O antigens of O groups 31 to 49, inclusive, were determined by means of OH sera and boiled culture. The use of boiled culture excluded the possibility of H antigen reaction. One should be aware, however, that *Proteus* O sera prepared by a culture heated at 100° C for 2½ hours may possess H agglutinins up to titers of 1:320. This was our experience when strains were used which occurred in a certain morphological form of variation to be described in detail in a subsequent paper. If the broth culture of a strain with this morphological form of variation after treatment by boiling was washed once with saline and afterwards suspended in the same volume of saline, an antiserum prepared from such an antigen would not contain H agglutinins. Boiling at about 125° for 2 hours likewise excluded the possibility of H antigenicity.

O absorptions were performed as follows: bacteria from a 20-hour agar plate were suspended in saline heated to 100° C for 1 hour and centrifuged. In cases where the supernatant fluid looked cloudy the bacteria were washed once in saline and again centrifuged. Serum diluted 1:20 and bacteria were mixed well, incubated 2 hours at 37° C, and put overnight in the refrigerator; thereafter the mixture was centrifuged and the procedure repeated, as one absorption proved not to be sufficient in all cases.

When dealing with the preparation of pure H sera the O agglutinins were removed by one absorption with the homologous culture treated by boiling for 2 hours at 100° C.

H absorptions were always made with a living 20-hour agar plate culture. The culture was suspended carefully in serum diluted 1:25 or 1:50, the suspension incubated 1—2 hours at 37° C, then centrifuged and a new absorption made. The mixture was kept overnight in the refrigerator. After the final centrifuging 1 drop of a 0.5 % formalinized salt solution was added as a preservative for each cc. absorbed serum.

The reading of O tube agglutination followed after 20 hours in a waterbath at 50° C; that of H tube agglutination after 4 to 6 hours, i. e., if the agglutination was negative or weakly positive after 4 hours, the reading was repeated after 6 hours. Some cultures kept more than 6 hours in waterbath may give rise to non-specific and misleading results even if pure H sera are used.

These uncertainties in the H antigenic determination can be escaped if, instead of tube agglutination one makes use of slide agglutination

with pure H sera in a dilution of 1: 50 or 1: 100, and living agar culture. The serum dilution will depend upon the titer of the particular serum. In the performance of the slide agglutination a bit of growth is emulsified in two drops of serum and agglutination becomes apparent after the slide is tilted back and forth for about half a minute. This H agglutination test is recommended as the most reliable for *Proteus* H antigen determination. The pure H serum (H serum absorbed with boiled culture) can be preserved in the refrigerator for months.

Because of the previously mentioned morphological form of variation, which under certain conditions may cause an O inagglutinability of the living and formalinized culture, slide agglutination with living culture can not be recommended for the determination of O antigens. In many cases good results can be obtained, but for uniform results tube agglutination is the test of choice.

In the typing of *Proteus* strains according to the extended antigenic table, attention must be called to another form of variation which has already been described by Kauffmann and Pereh for the X strains. This variation takes place in both O and H antigens and is apparent from the fact that a strain may have a partial antigen more or less developed. Thus different sera prepared from a series of subcultures of the same strain may produce variable agglutinins for a particular antigen. This variation may range from good to poor, to completely negative. It appears to occur in many antigens of the extended schema and may give rise to error in the complete type diagnosis. The extent of such variation is unknown at present.

Investigative Results.

1. Simplified Schema.

The results of the investigations undertaken will be found in summarized form in the tables. By reference to table 2, which contains the simplified antigenic schema for *Proteus*, it will be seen that 49 O groups were differentiated from our collection of 545 strains of *Proteus* organisms. Seven strains only of the 545 did not fall in the 49 O groups. Five of these were spontaneously agglutinable and did not permit a reliable O antigen determination, while two strains contained antigens which excluded them from the 49 O groups.

It will be noted that the distribution of strains by O groups is far greater in groups 1 to 30, than in groups 31 to 49. In fact, 87 per cent of all strains were grouped by O sera 1 to 30.

By means of 3 H sera it was possible to type, according to the simplified schema, 79 per cent of all strains, while 19 H sera were necessary to type the whole material. The simplified table contains 72 types distributed among 30 different O groups and 98 types distributed among the 49 O groups which have been distinguished. It is readily seen, and has often been stated in the literature, that the

variety of O antigens is much greater than that of H antigens. This is born out by the investigations on *Proteus* just as it has been established for *Escherichia coli*.

2. *Extended Schema.*

A careful investigation has been made of all the O and H partial antigens in the *Proteus* types of the first 30 O groups. The data are presented in Table 1. This work represents an analysis of 473 strains from which 79 types were differentiated. A total of 55 partial O antigens were distinguished in the 30 O groups and 31 H partial antigens. Again the greater variety of O partial antigens is marked in contrast to H antigens.

Biochemical Investigation.

The cultures were examined in the following substrates:

1. Broth containing 0.5 per cent of the respective carbohydrates adonitol, dulcitol, sorbitol, l-arabinose, xylose, l-rhamnose, maltose, salicin, inositol, lactose, sucrose, mannitol and glucose.

2. Ferrochloride-gelatin for determination of H_2S and gelatine splitting; liquid urea; Simmons' glucose agar and Simmons' citrate agar; caseinbroth for indol production; special broths for determination of KNO_3 reduction and production of the Voges-Proskauer and methylred reaction.

Two cultural types of *Proteus* were distinguished among the organisms of our collection which are referred to in Tables 1 and 2 as »Cultural Type 1« and »Cultural Type 2«. Cultural Type 1 as a rule produced acid rapidly in xylose, maltose, salicin and sucrose; it regularly formed indol. Cultural Type 2 refers to cultures which did not ferment maltose, produce indol, and gave either a delayed or negative reaction in salicin and sucrose.

As a rule all strains fermented xylose rapidly, produced acid and gas in glucose, liquefied gelatin, formed H_2S , hydrolyzed urea and failed to ferment l-rhamnose. With but single exceptions all strains hydrolyzed urea promptly and reduced nitrate to nitrite. Most strains were methylred positive and with but single exceptions they did not produce acetylmethylcarbinol. Any deviations from the rule are noted in a column of the tables under »Special Remarks«.

It will be seen from Table 1 that there is a relationship of H antigens to cultural type. All strains in the extended schema possessing the H antigens 1 a, 1 b, 1 c (X 19) and 1 a, 1 b, 1 d (XL) belong to cultural type 1. Any deviations from this rule apply either to deviations in the structure of H antigens 1 a, 1 b, 1 c or 1 a, 1 b, 1 d, or to H antigens of higher numbers.

All strains in the extended schema (Table 1) possessing the H antigens 1 a, 1 e, 1 e (XK), H 2, H 3, H 4 and H 13, a. o., belong to cultural type 2. One exception should be noted, namely 1 strains (F 63)

Table 1.
Diagnostic Proteus Antigenic Schema (extended)
according to Beat Perch.

O Group	Test Strain	Antigens		Number of Cases	Cultural Type	Special Remarks
		O	H			
1	X 19 XL	1a 1a, 1b	1a, 1b, 1c 1a, 1b, 1d	2 5	1 1	Xylose + or —
2	X 2	2a	1a, 1b, 1c	3	1	
3	XK F 403 F 248	3a, 3b 3a, 3b 3a, 3b	1a, 1c, 1c 2a, 2b, 2c, 2f 2a, 2c, 2c	8 10 2	2 2 2	
4	U 8 F 407 F 394	4a, 4b 4a, 4c 4a, 4c	1a, 1b, 1d 8a 16a	6 1 1	1 1 1	Xylose + or — " —; salicin —; urca — " —; " —
5	F 16 F 196 F 267	5a, 5b 5a, 5c 5a, 5c	1a, 1c, 1c 1a, 1c, 1c 3a, 3b ...	3 2 4	2 2 2	
6	F 181 F 78 F 116	6a 6a 6a	1a, 1c, 1c 2a, 2b, 2c, 2f 3a, 3b ...	3 17 18	2 2 2	
7	F 27 U 144 F 387	7a, 7b 7a, 7c, 11b 7a, 7b	1a, 1d, 1c, 1f 3a, 3b ... 4a, 4b, 4c	11 1 1	2 2 2	H ₂ S ×
8	F 30	8a	1a, 1b, 1c	4	1	
9	F 62 F 75	9a 9a	1a, 1c, 1e 2a, 2c, 2c	4 2	2 2	
10	F 39 F 2 F 73 P 506 F 280	10a 10a 10a 10a 10a	1a, 1c, 1c 2a, 2c, 2c 3a, 3b ... 4a, 4b, 4c 5a	13 22 10 6 5	2 2 2 2 2	
11	F 47 F 67 F 1 P 81 F 322	11a, 11b 11a, 11b, 11c 11a, 11d 11a, 11d 11a, 11b	1a, 1c, 1c 2a, 2b, 2c, 2f 2a, 2b, 2c, 2f 3a, 3b ... 6a	3 2 7 5 2	2 2 2 2 2	
12	F 65 F 358	12a 12a	1a, 1b, 1c 2a, 2c, 2e	9 6	1 2	
13	F 95 F 427 F 151 F 219	13a 13a 13a 13a	1a, 1c, 1c 2a, 2c, 2c 3a ... 4a, 4c, 4d	13 1 7 4	2 2 2 2	
14	F 120 S 127	14a, 14b 14a, 14c	1a, 1c, 1c 3a, 3b ...	1 2	2 2	
15	F 121 F 295a	15a 15a, 15b	1a, 1b, 1d ... 7a	3 1	1 1	Rham. + ² ; salicin + ⁷ Salicin + ⁸

Table 1 (continued).

O Group	Test Strain	Antigens		Number of Cases	Cultural Type	Special Remarks
		O	H			
16	F 55	16a	1a, 1c, 1e	6	2	
	P 206	16a, 16b	9a	1	2	
	F 485	16a, 16b	14a	16	2	
17	F 92	17a, 17b	1a, 1c, 1e	2	2	
	F 119	17a, 17c	10a	1	1	Salicin ×
18	F 136	18a	1a, 1c, 1e	1	2	
19	F 313	19a, 19b, 19c	1a, 1b, 1c	8	1	
	F 434	19a, 19c, 19d	1a, 1c, 1e	2	2	
	F 311	19a, 19c	3a, 3b ...	1	2	
	U 349	19a, 19b, 19c	11a	1	1	Salicin + ⁺
20	F 475	20a	1a, 1c, 1e	2	2	
	F 382	20a	2a, 2c, 2e	1	2	
21	M 205	21a	1a, 1b, 1c	3	1	
22	F 233	22a	1a, 1b, 1c	1	1	Xylose —
23	F 162	23a, 23b	1a, 1c, 1e	3	2	
	F 431	23a, 23c, 23d	2a, 2b, 2e, 2f	1	2	
	F 63	23a, 23c	2a, 2d, 2f	1	1	Salicin —
	F 45	23a, 23c, 23d	3a, 3b ...	1	2	
	F 296	23a, 23c	12a	1	1	Xylose + ⁰ ; salicin ×
24	F 288	24a	1a, 1c, 1e	9	2	
	F 103	24a	3a, 3b ...	4	2	
	F 90	24a	4a, 4b, 4e	4	2	
	F 330	24a	13a	2	2	
25	F 276	25a	1a, 1b, 1c	1	1	
26	F 58	26a, 3b	2a, 2c	5	2	
	F 458	26a, 3b	3a ...	3	2	
	P 372	26a, 3b	6a	1	1	
27	F 25	27a	2a, 2c, 2e	8	2	
	U 501	27a	3a, 3b ...	4	2	
28	U 509	28a	2a, 2c, 2e	12	2	
	F 87	28a	3a, 3b ...	7	2	
29	F 10	29a	13a	26	2	
30	F 384	30a	1a, 1c, 1e	1	2	
	F 29	30a	2a, 2b, 2e, 2f	9	2	
	F 321	30a, 30b	2a, 2c, 2e	1	2	
	F 152	30a	4a, 4b, 4e	1	2	
	U 96	30a	13a	2	2	
	F 49	30a	15a	5	2	

Key: ... means further antigens may be present, but not investigated
+² means positive after two days
— means negative after 30 days
× means delayed positive, often negative reaction

Table 2.
Diagnostic Proteus Antigenic Schema (Simplified)
according to F. Kauffmann and Beate Perch

O Group	Test Strain	Antigens		Number of Strains	Cultural Type	Special Remarks
		O	H			
1	X 19	1	1	9 (7)*	1	
2	X 2	2	1	3	1	
3	XK F 403	3	1	10 (8)	2	
		3	2	17 (12)	2	
4	U 8	4	1	8 (6)	1	
	F 407	4	8	1	1	Xylose —; salicin —; urea — " — " —
	F 394	4	16	1	1	
5	F 196	5	1	6 (5)	2	
	F 267	5	3	7 (4)	2	
6	F 181	6	1	3	2	
	F 78	6	2	22 (17)	2	
	F 116	6	3	25 (18)	2	
7	F 27	7	1	12 (11)	2	
	U 144	7	3	1	2	H ₂ S ×
	F 387	7	4	1	2	
8	F 30	8	1	4	1	
9	F 62	9	1	6 (4)	2	
	F 75	9	2	2	2	
10	F 39	10	1	19 (13)	2	
	F 2	10	2	28 (22)	2	
	F 73	10	3	11 (10)	2	
	P 506	10	4	6	2	
	F 280	10	5	5	2	
11	F 47	11	1	4 (3)	2	
	F 67	11	2	9	2	
	P 81	11	3	5	2	
	F 322	11	6	2	2	
12	F 65	12	1	13 (9)	1	
	F 358	12	2	7 (6)	2	
13	F 95	13	1	15 (13)	2	
	F 427	13	2	1	2	
	F 151	13	3	7	2	
	F 219	13	4	6 (4)	2	
14	F 120	14	1	1	2	
	S 127	14	3	3 (2)	2	
15	F 121	15	1	3	1	
	F 295a	15	7	1	1	Rham. + ² ; salicin + ⁷ Salicin + ⁸
16	F 55	16	1	7 (6)	2	
	P 206	16	9	1	2	
	F 485	16	14	18 (16)	2	

*) bracketed figures indicate the No. of cases.

Table 2 (continued 1).

O Group	Test Strain	Antigens		Number of Strains	Cultural Type	Special Remarks
		O	H			
17	F 92	17	1	2	2	Salicin ×
	F 119	17	10	1	1	
18	F 136	18	1	1	2	
19	F 313	19	1	11 (10)	1	Salicin + ⁷
	F 311	19	3	1	2	
	U 349	19	11	1	1	
20	F 475	20	1	2	2	
	F 382	20	2	1	2	
21	M 205	21	1	4 (3)	1	
22	F 233	22	1	1	1	Xylose —
23	F 162	23	1	7	2	1: Salicin — Xylose + ⁹ ; salicin ×
	F 431	23	2	2	1 and 2	
	F 45	23	3	1	2	
	F 296	23	12	1	1	
24	F 288	24	1	10 (9)	2	
	F 103	24	3	5 (4)	2	
	F 90	24	4	4	2	
	F 330	24	13	2	2	
25	F 276	25	1	1	1	
26	F 58	26	2	5	2	
	F 458	26	3	5 (3)	2	
	P 372	26	6	1	1	
27	F 25	27	2	12 (8)	2	
	U 501	27	3	4	2	
28	U 509	28	2	15 (12)	2	
	F 87	28	3	7	2	
29	F 10	29	13	32 (26)	2	
30	F 384	30	1	1	2	
	F 29	30	2	11 (10)	2	
	F 152	30	4	1	2	
	U 96	30	13	2	2	
	F 49	30	15	10 (5)	2	
31	F 110	31	1	4	1	
	F 125	31	2	9 (6)	2	
32	F 139	32	1	3	1	
	F 388	32	3	1	2	
	F 53	32	5	2	1	
33	U 510	33	3	7	2	
34	F 72	34	6	2	1	
35	F 335	35	2	2	2	
36	F 305	36	3	2	2	Rham. +; salicin + ¹¹
	F 398	36	7	1	1	

Table 2 (continued 2).

O Group	Test Strain	Antigens		Number of Strains	Cultural Type	Special Remarks
		O	H			
37	F 100b	37	17	1	1	Xylose + ⁵ ; salicin + ⁷
38	F 420	38	1	1	2	Xylose + ⁵
	F 158	38	2	2	2	
39	F 105b	39	18	1	1	
40	F 386	40	4	2	2	
41	F 409	41	1	5 (4)	2	
	P 522	41	2	2 (1)	2	
42	F 163	42	1	3	1	
43	F 433	43	2	3 (2)	2	
44	F 108	44	11	1	1	Rham. +; salicin ×
	F 179	44	19	1	1	» + » —
45	F 171	45	11	1	1	Salicin ×
46	F 223	46	17	1	1	Salicin ×
47	F 285	47	1	3 (2)	1	
48	P 368	48	1	1	2	
49	F 389	49	2	1	2	

which was reckoned to have antigen H 2 but had an additional special factor. This strain belonged to cultural type 1; certainly it gave a delayed or negative reaction in salicin.

Phase Variation.

Since the serology of the *Proteus* group is a parallel to that of the *Salmonella* group, it seemed well to investigate the possibility of phase variation. While there were no disturbing overlapping reactions among the H antigens which might lead one to believe in such a phenomenon, on several occasions in specimens from the same person we encountered strains from the same O groups which contained different H antigens. Tentative experiments were therefore undertaken with certain of our strains by the Gard plate method.

Positive evidence of phase variation was not obtained in all of our experiments, but it will be seen from Table 3 that with three strains bearing 3 different H antigens a second phase was induced. Attempts were also made to have the second phase revert to phase 1, but without success. The phase 2 obtained in these experiments may be regarded as a so-called artificial or induced phase. Whether a »natural« phase variation does occur is still an open question and subject to further studies.

Table 3.
Phase variation.

Strain	O Antigens	H Antigens	
		1. Phase	2. Phase
181	6a	1a, 1c, 1e	1, 2, 5
78	6a	2a, 2b, 2c, 2f	1, 3, 4
144	7a, 7c, 11b	3a, 3b...	1, 2, 4, 6

Key: numbers 1, 2, 3 in column 2. phase are not identical with the corresponding numbers in column 1. phase.

Examination of Strains of S. Winkle.

When the experimental studies cited above were finished, the paper of S. Winkle, entitled »Zur Typendifferenzierung in der Gattung *Proteus* Hauser« was called to my attention. A *Proteus* antigenic table is presenting in this work, in which Winkle has established 12 different types involving 13 different O antigens and 8 different H antigens. The antigenic schema of Winkle is reproduced in Table 4.

I am greatly obliged to Dr. Winkle for having placed his test strains at my disposal. The results of the preliminary orienting investigations with his strains and our sera were as follows:

Strain A was strongly agglutinated in serum of our O group 3 (XK) and strain B was strongly agglutinated by serum of our O group 26; these 2 strains could therefore be reckoned to O group 3 and 26, respectively.

Winkle's strains C, D and E were agglutinated by 3 different sera of our O group 23, representing 3 subgroups and therefore regarded as members of this O group 23.

Table 4.
Antigenic Table according to S. Winkle.

Sero Types	Test Strains	O Antigens	H Antigens
	X ₂	I (II)	a b c
	X ₁₀	(II) III	a b c
	XL	III	a c e
	XK	IV V	a b d
A	390	IV VI	a b d
B	247	V VII	f
C	383	VII VIII	a b d
D	653	VIII IX	a b d
E	47	VIII X	f
F	397	XI	a b c
G	52	XII	g
H	56	XIII	h

Strain F was strongly agglutinated by serum of our O group 12. The strains G and H were agglutinated by our sera of O groups 32 and 42, respectively, although the reactions did not reach the titers.

The H antigens a, b, c — a, b, d — a, e, e of Winkle correspond to the partial antigens designated H antigen 1 (Kauffmann and Perch). Winkle's antigen f is similar to our H antigen 2, while the g antigen corresponds to none of our H antigens. The h antigen of Winkle was found to be similar to H antigen 18 of Kauffmann and Perch.

It is, however, emphasized that these investigations are based only upon a unilateral agglutination of our H immune sera with Winkle's strains, and the same is true of the O agglutination tests. Detailed comparative studies shall be accounted for in the afore-mentioned greater publication.

Discussion.

Kauffmann and Perch set the pattern for the present work with their paper on the occurrence of Proteus X Strains in Denmark (1 and 2). An antigenic table was published in simplified form (1) for Proteus O groups 1 to 6, while both a complete and simplified formula were given for the X strains (O groups 1 to 3).

The present work is a considerable extension of those studies. A »complete« antigenic table in elaborated form has been established for the first 30 O groups, while a simplified antigenic table has been prepared for 49 O groups. Although the »complete« antigenic table appears complex in comparison to the simplified schema, it must be stressed that all Proteus antigens are not contained in the »complete« antigenic table; only the important antigens are involved. The simplified schema is a modification of the »complete« antigenic table; in the former only the *diagnostic* important antigens are presented.

The simplified Proteus schema might be likened to the antigenic tables which have been established for the Salmonella and Coli groups, in that it is a diagnostic table for determination of types, not a list of all existing antigens. It is further emphasized that only the diagnostically important O and H antigens were considered *as they occurred under natural conditions*.

A few preliminary investigations were made on the possibility of phase variation within the H antigens. These investigations indicate that in some cases it is possible to isolate artificial or induced phases, by the Gard technique, on agar plates containing homologous H immune serum. These attempts are not concluded, and further work will be carried out with the H antigens.

The same is true for the comparative studies made with the strains reported by Winkle. These were received at the end of my experimental work.

The chief purpose of the present paper has been to publish antigenic tables, which from the year 1944 have been the subject of

systematic investigations. A more detailed report of all results obtained will be reserved for a subsequent monograph on the serology of the *Proteus* group.

Summary.

A report is given of diagnostic antigenic tables of the *Proteus* group, partly in a complete state (with 30 O groups) and partly in a simplified form (with 49 O groups). The division of O groups into types was accomplished by means of H antigens. A total of 98 types is reported in the simplified antigenic table from a study of 538 strains.

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STUDIES ON THE AGGLUTININS AGAINST HEMOLYTIC STREPTOCOCCI IN RHEUMATIC DISEASES

By *Sten Winblad and Gunnar Edström.*

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As known, hemolytic streptococci grow in chains, and it is this arrangement that is considered responsible for the readiness with which these bacteria spontaneously agglutinate. Recent investigations have shown that this spontaneous agglutination is due to the structure of the hemolytic streptococci (*Thulin*). For instance, if these streptococci are encapsulated, neither spontaneous agglutination nor agglutinins will appear as anti-bodies. If however they are not encapsulated, spontaneous agglutination will generally occur, except in older cultures which are as a rule non-agglutinable and which therefore may be used as an antigen in agglutination reactions intended to show an anti-body in the form of an agglutinin.

Griffith has demonstrated how a type-specific factor may be shown in immune sera after the absorption of agglutinins, and how agglutination may then be used for identification purposes in determining the streptococcal type.

It is, however, evident that not all the agglutinins are type-specific, some being only group-specific or at least »type non-specific« such as those agglutinins against hemolytic streptococci encountered in serum from patients with rheumatoid arthritis. *Nicholls* and *Stainsby* (1931) were the first to observe such agglutination, but against a culture of alpha-hemolytic streptococci. They expressed the opinion that this streptococcal strain was the only one against which such agglutination could be demonstrated. *Dawson* and his corroborators could show an agglutination also in the serum taken from patients with rheumatoid arthritis, but only if — hemolytic streptococci were used as antigen in the test. Furthermore they observed that various different strains of

such cocci could be used for this reaction and were able to show agglutination in 69 per cent of their cases (the minimum positive titre approved being 1/160). In clearly established cases of streptococcal infection without rheumatic complications they found positive agglutination in 8 per cent of the cases only. A technically important detail was recognised by these researchers, to wit, that streptococcal strains must be living and have grown not more than 14 hours, if they are to be usable in the reaction.

Other workers have since then confirmed these observations. *Keefer, Myers* and *Oppels* demonstrated positive agglutination against hemolytic streptococci in the sera of 54.5 per cent of a rheumatoid arthritic material, this result agrees more or less to those published by other investigators (*Cox & Hill; McEwen, Alexander* and *Bunim; Levinthal; Thomas; Coss* and *Boots; Packalén* and *deGara; Kalbak*). Of these researchers *Kalbak* showed that in his material consisting of clearly established cases of rheumatoid arthritis no fewer than 95 per cent showed agglutination in a dilution of 1/20 or more. Positive agglutination in rheumatic fever or acute infection of hemolytic streptococci was less often (10 per cent) observable in his material. *Kalbak* applied *Dawson's* technique and used living streptococci as antigen in his reaction experiments.

Later investigations have, however, shown that *living* hemolytic streptococci are not the only ones that can be used in such agglutination tests for demonstrating agglutinins in the sera of patients with rheumatoid arthritis. *Thulin* has shown that *autoclaved* hemolytic streptococci, grown on pancreatin digested broth, may just as well be used as an antigen in this reaction. Such agglutination is also manifestable in the serum from patients with rheumatic fever or nephritis. In accordance with the system of terms adopted for *Salmonella* serology *Thulin* gave the name of O-antigen (body-antigen) to this streptococcal antigen. By means of absorption experiments he was able to show that this antigen is of a different nature from that in the above-mentioned streptococcal strains which were agglutinizable by the serum from rheumatoid arthritic cases only. There are evidently two naturally different antigens from hemolytic streptococci that give rise to agglutinins in rheumatoid arthritis. This argues in support of the assumption that hemolytic streptococci are in some way or other correlated not only to rheumatic fever but also to rheumatoid arthritis, but the exact mechanism of this relationship is probably complicated.

Of late years observations have been published concerning the sera from rheumatoid arthritic patients. The relevant importance of these observations is of the first order, inasmuch as they show that not only streptococci but also other bacteria as well as non-bacterial particles may be agglutinated by such sera. *Wallis* found that these sera will

also agglutinize decapsulated pneumococci; he also asserts that even particles of collodium will become clumpy if brought into contact with these sera. Even sensibilized blood cells of sheep may sometimes be agglutinated (*Waaler*). How such observations ought to be interpreted is hard to say, but they do direct our attention more than ever to this agglutination phenomenon and solicit a further study of the serology of this disease.

Serologically the above mentioned agglutinins against hemolytic streptococci differ from ordinary antibodies inasmuch as they generally occur in a disease so pronouncedly chronic from the very beginning as rheumatoid arthritis but not in acute streptococcal diseases. Owing to this characteristic other serological experiences will be of but little help in finding a feasible explanation of this phenomenon. It would, however, be interesting to determine the actual time these agglutinins appear in the serum. In an endeavour to find an answer to this question we have, as will be reported herebelow, examined the agglutination not only in rheumatoid arthritic serum but also in that from patients with rheumatic fever. We know very well that many cases of the last-mentioned disease are inclined to take a chronic course and that several cases actually develop later into rheumatoid arthritis. In view hereof we thought it possible in such cases to show the existence of agglutinins as are not demonstrable in the acute stage of the rheumatic disease.

The term L-agglutination.

In the experiments described herebelow only agglutination against living hemolytic streptococci has been employed. The antigen factor in this reaction is, as mentioned further up, different from the O-antigen obtained by autoclaving. A further characteristic of this factor is also that it is demonstrable only in certain strains of hemolytic streptococci that have no capsules and that nevertheless do agglutinate spontaneously. Furthermore, this antigen disappears in living streptococci if the strain is allowed to grow more than 14 hours, shaken or centrifuged (*Kalbak*). In view of these last mentioned facts it seems quite credible that the antigen factor is located on the surface of the bacterial body and is therefore easily removable. This has also been shown in a photographic reproduction (*Thulin*). As a suitable name for this antigen we suggest the designation »surface-antigen« or »living antigen« and »living agglutination« respectively because it is bound to a living culture. We decided upon the term L-agglutination not only for the above reason but also because it is adaptable for other languages (English: living; German: lebende). The term »surface-antigen« is hardly adequate, because also the capsular substance forms part of the surface of the bacteria.

Technique.

Culture: A culture of hemolytic streptococci group A, type 1, called SF 130 has been used in the reaction.

Broth I: 750 gm. finely minced beef is added to 1½ liters of water and placed in an ice chest where it is kept overnight. The following day this mixture is boiled 15 minutes, filtered through paper and alkalisied to pH 8.0 with 5 N NaOH. 10 gm. Neopepton and 4 gm. sec. sodium phosphate are then added per 500 ml. of the broth. The broth is re-boiled and its pH is adjusted to 8.2 after which it is again filtered through paper and autoclaved 20 mins. at 120 °C.

Broth II: Instead of the above mentioned Neopepton and sec. sodium phosphate, 10 gm. Bactopepton and 5 gm. NaCl are added to 1 liter of water (with a pH of 8.0). The broth is then boiled and its pH is adjusted to 8.2 after which it is filtered and poured into sterilized flasks which are then autoclaved at 120 °C. for twenty minutes. The pH is finally adjusted to 7.6—7.8.

Incubation: The broth I is amply inoculated with the culture and then incubated for 6 hours. 50 ml. of the broth thus incubated are then used for the inoculation of a flask of Broth II, which is in turn incubated 10—12 hours. This broth then constitutes the finished antigen.

Agglutination: Inactivated serum is diluted with 0.3 per cent NaCl, in a series 1/20—1/640. An equal quantity of antigen is then added to each of the test tubes of the series which are first incubated two hours in a water bath (52 °C) and then placed overnight in an ice-chest. The readings are always taken with the same magnification and the same strength of light. The agglutination is judged on the same principles as suggested by Kalbak; *Strength 0:* Completely diffused test. No agglutination. *Strength 1:* No real agglutination. When shaken the sediment will rise diffusely. *Strength 2:* The sediment falls into middle sized particles, the supernatant liquid being clear. *Strength 3:* The sediment falls into large integral pieces, the supernatant liquid is clear. *Strength 4:* The sediment forms a single large lump hard to shake to pieces. Strength 2, and upwards are considered positive.

As the antigen component in the reaction consists of a living broth culture, the value of the reaction is not always the same from one time to another. In order to keep the reaction as stable as possible a freshly prepared broth was used for 2—3 days only, after which new antigens were prepared. Moreover the sera used were tested repeatedly in order to avoid any doubt concerning their agglutination value. In spite of this precaution varying values are encountered, but this is seldom the case.

Material.

The material of the present study consists of 206 cases of rheumatoid arthritis. These cases were patients hospitalized at two rheumatic clinics (Rheumatic Clinic, Lund and Pensionstyrelsens Sjukhus, Malmö). Some of the patients were nursed at the Dept. for Internal diseases at the General Hospital of the town of Malmö. In nearly all cases tests were taken repeatedly and at various times. It was often possible to follow the agglutination titre a long time, the sedimentation rate being registered every week.

Results.

Normal Material. It was deemed important simultaneously to control the agglutination method by tests taken from normal material,

which consisted of serum from donors which was sent to the laboratory for routine control of Wasserman's reaction. The results of the agglutination determination of 105 normal sera will be apparent from Table 1.

Table 1.

No agglutination	Agglutination with dilution of at least						
	1/10	1/20	1/40	1/80	1/160	1/320	1/640
80	19	4	2	—	—	—	—
94,3 %		5,7 %					

From the above table it will be observable that 94.3 per cent manifested either no agglutination or agglutination in 1/10 dilution only, whilst 5.7 per cent showed agglutination in a 1/20 or higher dilution. In view of this result obtained from the control material we feel justified in considering 1/20 the lowest »positive« agglutination titre, as did *Kalbak*, and this limit has been used in the evaluation of the investigation material.

Rheumatoid arthritis. Of the above mentioned 206 cases with this diagnosis, care was taken not to include any patient who had fallen ill with rheumatic fever, because the agglutination titres of these patients are dealt with separately further down. The distribution of the maximum titres registered in these 206 cases of rheumatoid arthritis is given in Table 2.

Table 2.

No agglutination or agglutination 1/10	Agglutination with a dilution of					
	1/20	1/40	1/80	1/160	1/320	1/640
66	23	35	36	24	15	7
32,0 %	140 (68,0 %)					

Whilst 140 cases showed a positive agglutination (titre 1/20 or more), agglutination was absent in 66 cases. Thus two thirds of the patients exhibited elevated agglutination titre. The number of cases in each of the titre classes is rather uniform although it is somewhat less in the higher classes. Owing to the resemblance between our technique and that of *Kalbak* we could compare our results best with his, and found that our material exhibited a somewhat lower frequency of positive agglutination titres.

We also thought it might be interesting to examine the relationship between the maximum registered agglutination titre and the maximum erythrocyte sedimentation rate (E. S. R.) registered in the course of the disease. As the E. S. R. has since long been recognized as the best indicator of the intensity of this rheumatic disease, a comparison of the two above mentioned maximi may to a certain extent illustrate any correlation existing between the agglutination titres and degree or stage of the various cases. A comparison of this nature is illustrated in Table 3.

Table 3.

Maximal agglutination titre	Sedimentation rate mm/h					
	0-20	21-30	41-60	61-80	81-100	101-120
Negative agglutination	38	20	6	2	—	—
Positive agglutination 1/20	10	10	2	1	—	—
1/40	9	17	6	3	—	—
1/80	11	14	4	4	3	—
1/160	9	3	4	5	2	1
1/320	4	3	1	2	2	3
1/640	2	—	—	2	1	2

The above results prove the existence of a correlation between an elevated agglutination titre and elevated E. S. R. (This is 3.5 times the mean error, $r = + 0.244 \pm 0.0685$). It is thus usual for an elevated E. S. R. to be attended by an elevated agglutination titre and for a low E. S. R., by a low titre.

The antibody most typical of rheumatic fever, antistreptolysin, and the E. S. R. show a certain degree of parallelism (Winblad, Malmros & Wilander). We now find that there is a clear correlation also in rheumatoid arthritis between the E. S. R. and its typical anti-body, to wit, agglutination of this type. It has been suspected that the agglutination be due to the same disturbance in the balance of the plasma proteins as gives rise to an elevated E. S. R. (Packalén). Böni and Winblad have, however, shown that an elevated E. S. R. in chronic pulmonary tuberculosis is not attended by an increase in this type of agglutination. This means then that the elevation of the agglutination titre cannot be attributed to a non-specific cause such as a disturbance in the balance of the protein fraction. On the other hand, however, it is quite credible that also in *rheumatoid arthritis the production of antibodies increases with increasing intensity of the disease, as is the case with rheumatic fever.*

Rheumatic fever: Seventy-two cases of rheumatic fever were examined for agglutinins of this type. The onset of the disease in every case was in the form of rheumatoid arthritis, generally with prodromal

infections of the throat and upper air-ways. The stage to which the disease had advanced before these patients sought relief varied, however, from case to case. Thus some were examined in the acute phase of the disease (1st—2nd month), whilst others came later, and a few during both the acute and the later stages of the disease. As known, the course of rheumatic fever varies considerably in different patients. Whilst some escape with a slight lesion of short duration, it may be serious and long in others, and in some people even show a tendency to become chronic (formerly called secondary rheumatoid arthritis). Consequently, when judging this antibody in cases of rheumatic fever, we considered it necessary to pay special attention to the age of the disease at the time of the examination.

Table 4 illustrates the age of the diseases and at the same time gives the registered values of the agglutination and antistreptolysin titres of the patients.

Table 4.

Elapse after onset	Agglutination		Antistreptolysin titre	
	positive	negative	>200 Units	<200 Units
1st—2nd month.	4	20	18	6
3rd—4th "	5	11	13	3
5th—6th "	8	8	14	2
7th—8th "	3	6	5	4
> 8th "	4	3	4	3
	24	48	54	18

Whilst the antistreptolysin titre, as was to be expected, was generally elevated in these cases of rheumatic fever, positive agglutination was established only in altogether 24 cases of a total number of 72 (34.7 per cent). If, however, we observe the occurrence of positive agglutination titres in the various stages of the disease we find that such titres are comparatively uncommon during the first four months of the disease, but are observable in 50 per cent of the cases examined four months or more after the onset of the disease. *Kalbak* found in his material that only 10 per cent of the rheumatic fever cases demonstrated positive agglutination. This percentage probably refers to cases in the acute stage of the disease. From Table 4 it will be apparent that the number of cases per cent with positive agglutination increases with increasing age of the disease.

Those cases nursed for a long time do not of course belong to the least serious types of rheumatic fever, and those cases in our material that were examined over a long period after the onset of the acute

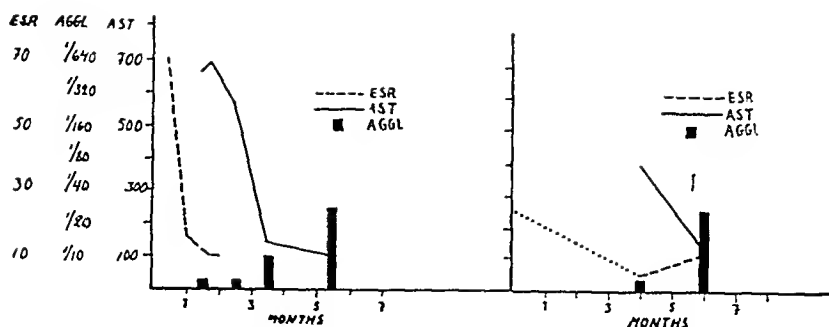


Fig. 1.

Case Ev. Er. (left) and case Ma. Ar. (right). Two cases of rheumatic fever. Increased agglutination titre manifestable in 5th month.

stage, also have a tendency to a prolonged course and sometimes to chronicity.

It is therefore presumably possible for a negative agglutination to become positive during the course of rheumatic fever. This change was observed in five cases in the course of the present investigation. All of these cases were patients who during the acute phase showed a high antistreptolysin titre with a tendency to drop and which in the course of the disease develops a positive agglutination not apparent in the acute phase. These cases are illustrated in Figs. 1—4. (Anamnesis of these cases, see below). *This positive agglutination titre becomes apparent in the 5th—7th month*, which, judging by the results of the material shown in Table 4, is at a time that might be expected.

As it is here a question of an antibody appearing relatively late in the disease, one wonders whether the patient has contracted something else that might explain the occurrence of a new antigen, which in turn has given rise to this new antibody. If this be the case, one might very well imagine that a new attack of tonsillitis, tonsillectomy of the extraction of a tooth might have been responsible for a fresh supply of hemolytic streptococci to the circulation. The two last men-

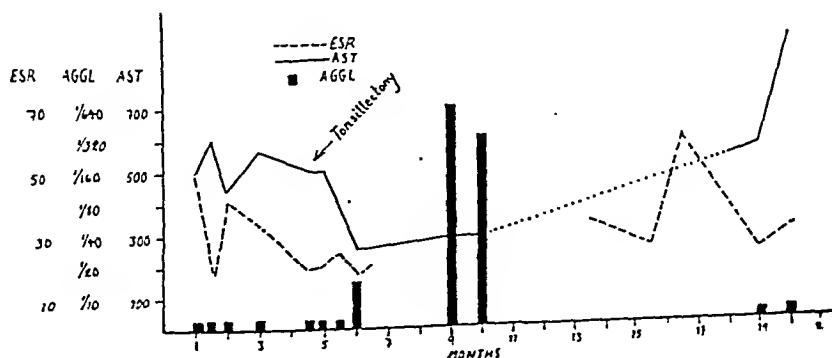


Fig. 2.

Case St. No. Prolonged rheumatic fever with increasing agglutination titre manifestable in 6th month. This agglutination disappeared later.

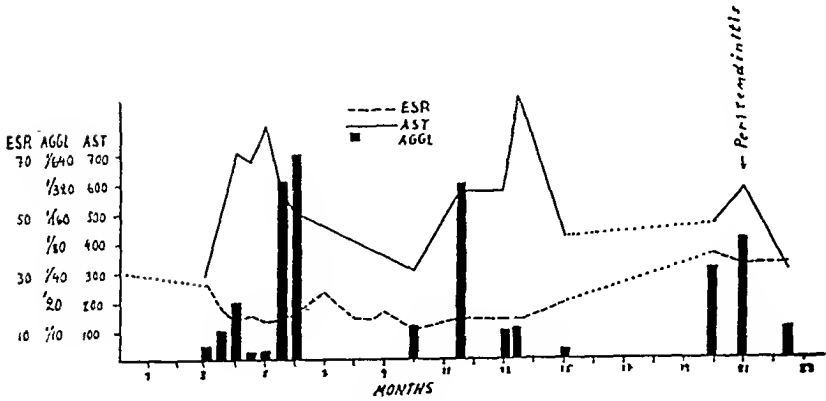


Fig. 3.

Case Ul. Fr. Typical case of rheumatic fever with positive agglutination as from 5th month.

tioned measures are common treatment for the elimination of chronic infection nests in these patients, and what is more the operations are as a rule performed a few months after the acute phase has passed over. It was only in one of our five cases (Fig. 2) that tonsillecctomy had been performed immediately before the agglutination changed from negative to positive. In no other case was any such operation reported nor could the occurrence of any new infection at a time referable to the positive agglutination be established. The teeth in all the patients in question were in good repair and had needed but little treatment, and then not before the agglutination had already turned positive.

Another question is also worthy of consideration. Is this agglutination a phenomenon of habitually late appearance, a long time after ordinary infections with hemolytic streptococci even without the occurrence of a rheumatic complication? An adequately large material of cases of uncomplicated streptococcal infections could of course give an answer to this question, but it is hard to collect such material. It

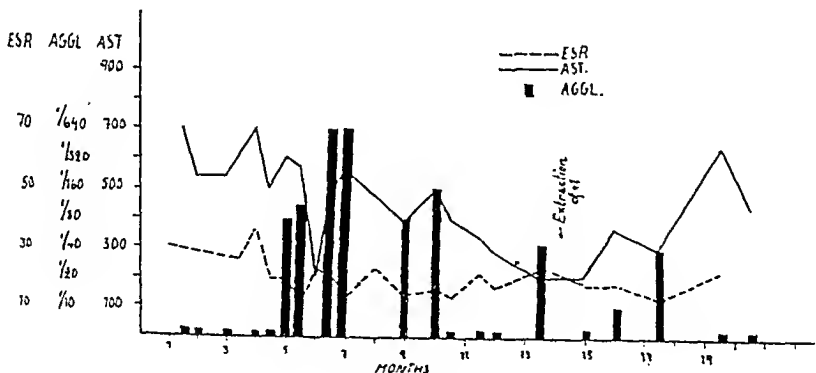


Fig. 4.

Case Bi. Sv. Typical rheumatic fever with increasing agglutination titre after the acute stage.

would seem, however, that the answer to the above question will hardly be in the affirmative because elevated agglutination titres are so seldom found in normal material in spite of the fact infections with these bacteria were common among the population during the time the present investigation was being made. In a large material sent for Wassermann's reaction *Kalbak* was able to show that this type of agglutination was an attendant of rheumatic diseases only.

We are thus able to conclude that *positive agglutination against living hemolytic streptococci occurs in $\frac{2}{3}$ of all cases of rheumatoid arthritis and in half of the cases of rheumatic fever examined four months or more subsequent to the onset of the disease.* Such agglutination, however, is rare during the acute phase of rheumatic fever.

Discussion.

The L-agglutination analysed in this investigation is thus characterized by the fact that it appears late in cases of rheumatic fever and otherwise only in such a pronounced chronic disease as rheumatoid arthritis. Serologically, it therefore differs from other antibodies inasmuch it does not become apparent until a long time after the onset of the disease.

The reason for such a late appearance may be sought in several possibilities. We have already mentioned two, viz., that a new infection or an operation such as tonsillectomy or the extraction of a tooth might be responsible for the production of a new antigen at a time referable to the retarded agglutination, secondly, that this agglutination might be a regularly late phenomenon in hemolytical streptococcal infections. But for reasons mentioned further up, however, it seems as if neither of these explanations can be accepted.

Three more possibilities might be discussed.

1. The agglutination in question might be due to the individual being a chronic bacillary host. After infections with *Salmonella typhi* (typhoid fever) the patient may become a chronic host of this nature and in this condition he will show an agglutination especially against Vi-antigen, an agglutination appearing only late after the primary infection. Analogically this agglutination against hemolytic streptococci means that the patient is a bacillary host of hemolytic streptococci. *Kalbak* pointed out that an intravenous injection of living or killed hemolytic streptococci will cause a greater production of antibacterial antibodies, such as agglutinins, than antitoxic antibodies, such as antistreptolysin. As far as rheumatic diseases are concerned the assumption of a continuous bacterial activity has been indefensible as the essential factor responsible for the rheumatic disease, because neither sulphatherapy nor penicillin will produce any alleviation. But this is no evidence of the agglutination not being ascribable to the existence of residual hemolytic streptococci in the body, it still being uncertain whether the existence of agglutinins or survived hemolytic

streptococci is necessary for the continuation of the rheumatic condition.

2. The agglutination might possibly be explained by the assumption that the hemolytic streptococci change in such a manner during the disease that they develop a new antigen that does not appear in the acute stage but only after the elapse of some time and that this new antigen in turn stimulates the arisal of L-agglutinins. Examinations in vitro showed that only certain strains were suitable for this agglutination reaction, because they not agglutinate spontaneously unless they are encapsulated, in which case they are void of agglutinability (Thulin). Preliminary experiments (Winblad) showed that laboratory cultures grown on but poor substrate are occasionally usable as an antigen in the reaction. In other words it would seem as if these cultures, when grown on meagre media exudated a surface-antigen that is responsible for this reaction. Recently Thulin succeeded in obtaining an electron-micrograph of the substance which, located on the surface of the culture SF 130, is probably the active antigen factor. In analogy with these experiments in vitro it is feasible that the hemolytic streptococci may during the period they survive in the organism after an infection, exudate this superficial antigen, which in turn gives rise to the L-agglutinin. So far, this is of course to be considered only a hypothesis for future studies.

3. A further possibility is, as pointed out in the preamble, that the L-agglutination is not an antigen-antibody reaction but a non-specific flocculation phenomenon. This theory is brought forward by Wallis on the basis of agglutination experiments with decapsulated pneumococci and colloidium particles. Further experiments are, however, necessary before this obviously bold theory can be accepted.

L-agglutination absorbs much interest in the discussion of the genesis of rheumatoid arthritis. It may to a certain extent increase the diagnostical knowledge available for clinicians when diagnosing cases with chronic arthritic trouble. Its application is, however, limited by the fact that only $\frac{2}{3}$ of the cases show positive agglutination titres. This type of agglutination is of great interest for continued theoretical research work on serological reactions in rheumatic diseases because it stimulates to attack the problem from a new angle.

Casuistics of the cases illustrated in Figs. 1—4.

Ev. Er. (Fig. 1). Male. 20 yrs. Örebro. Las. Med. dept. J. N. 1294/46. Treated 22/5—31/5/46. Diagnosis: Febris reumatica. Fell ill without preceding infection 6 weeks before admittance for pains, swelling and stiffness of left knee joint. Right knee and both ankles gradually gave trouble, which had, however, partially ceased some time before admittance, when the trouble was limited to the knee joints. At onset E.S.R. was 70 mm/h. On admittance it had sunk to 16 mm/h. Status on hospitalization: Clear signs of endocarditis. Anti-streptolysin titre (AST), agglutination (Aggl.) and Erythrocyte sedimentation rate (E.S.R.): see Fig. 1 (left).

Ma. Ar. (Fig.). Female. 31 yrs. Rheum. Dept. Lund. Treated 4/10—12/12/46.

Diagnosis: Febris rheumatica c. artritide. Fell ill 4/5 with inflammation of the throat and high fever. 17/5 high fever again and shivering as well as aches in the whole body. 18/5 she developed pains in her right ankle and later also in the toe joints of the right foot and in the right knee joint. Temp. then 39.6 °C. Soon afterwards other joints were also engaged. Treated at another hosp. 7/6—17/6/1946. E.S.R. then 22 mm/h. Tonsillectomy performed there. On admittance: Teeth in good state of repair. Tonsillectomy performed free of irritation. Normal pharyngeal mucous membrane. Both ankles and the joints of the small toe engaged. Improvement during hosp. sojourn. No operative treatment there. AST, Aggl. and E.S.R. see Fig. 1. (right).

St. No. (Fig. 2). Male. 20 yrs. Rheum. Dept. 17/1—13/7/1946. Diag: Febris reumatica c. artritide et endocarditide. Fell ill at end of March 1944 with fever and inflammation of the throat. Temp. 39.2 °C. Treated at military hospital 24/4—23/9/1944 for febris reumatica. All joints free of pain on discharge. In Oct. 1945 again inflammation of the throat and high fever, and abscess in the throat. Immediately afterwards, slight acute pains in the wrist-, ankle-, and shoulder-joints. Again admitted to Military hosp. until 20/12/45 when he was sent home until he could be admitted to a Rheum. Clinic. E.S.R. was then 45 mm/h. admitted at above Dept. 17/1/46. Was then ill, tired and weak. Pharyngeal mucous membrane was slightly granulated and his tonsils were enlarged and hypertrophic but without suppuration. Teeth in good state of repair. Auscultation: prolonged systolic murmur and a pre-systolic murmur over left I₃. Dorsally to the capillitium protuberances the size of a hazel nut and of the nodular type were palpated. Ankle-, shoulder-, elbow-, wrist-, and finger-joints were engaged. Peri-edematous swelling around the joints of the foot, toe and wrists. ASL, Aggl. and E.S.R., see Fig. 2.

Ul. Fr. (Fig. 3). Female. 23 yrs. Rheumatic Clinic. 8/4—13/7/1946. Diagnosis: Febris reumatica c. endocarditide. Valvular lesion. For a short time in Juli 1945 the patient had a swollen right ankle. Fell ill in beginning of Dec. 1945 with a chill, cold in the head and a sore throat as well as with nodular rash on the legs. In the middle of Dec. the left tarsus began to swell. After a few days the knee joints and the right ankle were also engaged. E.S.R. was then 30 mm/H. Tonsillectomy in 1932. Status on admittance: teeth in good state of repair, throat normal, and clean tonsil bed. In left sub-mandibular angle a lymphadenitis. Over the heart harsh systolic murmurs with punct. max. in left I₃ adjacent the sternum. Joints somewhat tender. During hosp. sojourn gradual improvement. ASL, Aggl. and E.S.R. see Fig. 3.

Bi. Sv. (Fig. 4). Female. 22 yrs. Rheum. Clinic. 4/4—13/7/1946. Diagnosis: Febris reumatica c. artritide. Valvular lesion. In Dec. 1944 the patient had inflammation of the throat, fever and an E.S.R. of 50 mm/h was registered. The joints had, however, given the patient no trouble at all. In Jan. 1945 mitral stenosis was established. 15/1/46 the patient fell ill with fever and tenderness over the left wrist joint. E.S.R. 30/mm/h. Then fever and pains in several joints which swole. On admittance: Teeth in good repair, normal throat and no palpable lymphadenitis. Auscultation: systolic and diastolic murmurs. Right wrist-, and right finger joints were engaged. Symptoms gradually decreased. 13/10/47 new attack of pains in left wrist joint with peritendinitis over back of hand. ASL, Aggl. and E.S.R., see Fig. 4.

Summary.

The designation L-agglutination is suggested for the agglutination against living hemolytic streptococci and observable in the sera of patients with rheumatoid arthritis, as distinguished from O-agglutina-

tion, which is the agglutination against antoclated hemolytic streptococci.

Of 206 cases of chronic polyarthritis 140 (68 per cent) showed positive L-agglutination (in a dilution of $\frac{1}{20}$ or more), such agglutination being observable only in 5.7 per cent of a normal material.

An actual corrolationship was discernible between the E.S.R. and the agglutination titre, elevated E.S.R. generally being attended by high agglutination titre.

Of 72 cases of rheumatic fever 24 (34.7 per cent) showed positive agglutination. Of these cases positive agglutination was, however, rare during the first four months but occurred in about fifty per cent of the cases after this elapse. Cases of rheumatic fever tending to be prolonged and become chronic often showed agglutination, which, however, did not begin to appear until 4—7 months after the onset of the disease.

L-agglutination is characterized by the fact that it occurs only in rheumatoid arthritic patients and in the late stage of rheumatic fever. Various possible explanations of this antibody and its retarded appearance are discussed. The most feasible explanation seems to be that survived hemolytic streptococci form a new superficial antigen on the body of the bacteria and that this antigen stimulates the production of the antibody exhibiting itself in the form of L-agglutinins.

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THE BLOOD GROUP SYSTEM L. A NEW BLOOD GROUP L_2 . A CASE OF EPISTASY WITHIN THE BLOOD GROUPS.

By P. H. Andresen.

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In a previous paper¹⁾ the blood group L has been described. By means of a serum »Anna Eriksen«^{*}) it has been demonstrated that the described anti-L quite corresponds to the anti-Lewis described by Mourant²⁾, so that the groups L and »Lewis« are identical.

Shortly after my above report had appeared, I succeeded in demonstrating an additional agglutinin, and even the first investigations showed the very near relation of the corresponding receptor L_2 to the receptor L, both these receptors never being found simultaneously in the same adult person. (Hereafter L (Lewis) will be designated L_1).

The first examinations are collected in *table I*. (As the man in question belonged to group A_1MN , and the serum contained a strong anti-B which was difficult to eliminate, these examinations have been made only on blood samples from persons of the groups O, A_1 , and A_2).

Table I.

Group:	L_1+L_2-	L_1-L_2+	L_1+L_2+	L_1-L_2-	Total
	105	300	0 (8*)	112	525
Adults:	(20 %)	(57.1 %)	(1.5 %)	(21.4 %)	
Children of					
0-12 months:	61	20	44	34	159
	(38.3 %)	(12.5 %)	(27.9 %)	(21.3 %)	

*) L_1 +, but weak.

*) Supplied me through the kindness of Dr. O. Hartmann, Oslo.

Table I shows the near relation between L_1 and L_2 , and the findings in the children show that L_2 has the characters which should be expected in the allele blood group character corresponding to L_1 ¹), the character not developing until rather late and dominating only in adults. However, the group L_1-L_2- gives rise to reflexion, as it could not be anticipated.

A close study of the occurrence of the group L_1-L_2- shows, however, that table I does not give a correct picture of its occurrence. It was soon discovered that the type O L_1-L_2- was much more rare than the type $A_1 L_1-L_2-$, and consequently it was necessary to divide the material according to the groups within the ABO system. From table II is seen the distribution of the characters L_2+ and L_2- within the groups A_1 , A_2 , and O.

Table II.

	L_2+	L_2-	Total
Group A_1	77 (41.8 %)	107 (58.2 %)	184
» A_2	52 (70.0 %)	23 (30.0 %)	75
» O	199 (72.0 %)	77 (28.0 %)	276
	328 (62 %)	207 (38 %)	528

Distribution of the characters L_2+ and L_2- within the groups O, A_1 and A_2 .

From the table it is evident that there must be some relation or other between the occurrence of the characters A_1 and L_2 , whereas no relation between the receptor L_1 and the other types has been found at the previous examinations.

As the groups A_1 and L_2 , no doubt, are inherited quite independently of each other, it is to be assumed that the finding may be accounted for by the fact that *the mere presence of the characters A_1 (or the corresponding gene) inhibits the development of the receptor L_2 , which corresponds to the phenomenon in genetics designated epistasy.*

The epistasy in question is, however, only *incomplete*, A_1 inhibiting the development of L_2 only in a certain number of cases. Once this phenomenon had been realized, recognition was attained that it was always just in persons of type A_1 that reactions with anti- L_2 were found which were weaker or which in some cases were uncertain.

After these observations had been made, it was deemed most reasonable preliminarily to continue only the investigations withing group O (even if conditions seemed to be quite similar in type A_2). The appearances in type B will be subjected to a special examination later.

In table III are recorded some investigations within group O, including only the cases examined after the mentioned epistasy had

been realized, and in which all cases of OL_1-L_2- have been subjected to a special examination.

Table III.

	L_1+L_2+	L_1+L_2-	L_1-L_2+	L_1-L_2-	Total
Group O	0	46 (20 %)	178 (74 %)	14 (6 %)	238
Distribution of the groups in the L system within group O.					

The table shows that the phenotype L_1-L_2- is found in group O, and repeated examinations in the same person have shown that this is a reality which has not been brought about by impairment of the blood corpuscles (transport etc.).

So far, it has not been possible to explain this phenomenon. Possibly it is a phenotypical aspect caused by an impairing factor (in analogy with the relations of L_2 to group A_1), possibly the group is due to a special allele gene; later investigations may reveal this. Already the isolated examination of the receptor L_1 showed that the phenotype differed very much in adults and infants. This is confirmed even by *table I*, from which it is seen that the lack of the receptor L_2 is much more frequent in children than in adults. This is also the case, even if only children of group O are taken into consideration. From a statement corresponding to *table II* it is seen that L_2+ is found in 48 per cent of children belonging to group O (72 per cent in adults); this phenomenon is still more pronounced, if only children between 1 and 6 months are considered.

In order to supplement the investigations in children, the blood from 50 newborn children has also been examined immediately after their birth, and here it proves that both the receptors L_1 and L_2 are so weak that they cannot be demonstrated by the usual technique. When much anti- L_1 serum is employed, the receptor L_1 can be demonstrated in some cases also in the newborn immediately after their birth.

The demonstration of the receptor L_2 has confirmed the theory advanced in my first paper on the receptor L_1 of the presence of an allele group character and thus corroborated the advanced theory of inheritance, but on account of the presence of group L_1-L_2- it is not yet possible to establish the inheritance with certainty.

The mentioned anti- L_2 was found in a man of group A_1MN who had never had any transfusion of blood, and it may be added that it has been possible to demonstrate anti- L_1 , in two men who had never had any blood transfusions.

Summary.

A new iso-agglutinin is demonstrated which reacts with a receptor designated L_2 , and which together with the previously described receptor L_1 forms a blood group system, the two receptors never being recognizable simultaneously in adults, L_2 dominating L_1 .

The lack of these two characters is demonstrated in about 20 per cent of the persons studied, but it is shown that in most cases this lack is due to an epistatic action of the A_1 gene against the receptor L_2 ; only in abt. 6 per cent of the cases the lack of both characters cannot be accounted for in this way.

Both receptors (L_1 and L_2) are absent or extremely weak at birth, but after that the character L_1 develops more rapidly than L_2 .

Both the iso-agglutinin anti- L_1 and anti- L_2 are found spontaneously not only in mothers, but also in men and do not demand any preceding immunization; in this respect they resemble anti-P and deviate from the rhesus agglutinins.

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ON ADENOLYMPHOMA

By William Kiær.

(Received for publication May 21st 1948.)

The term adenolymphoma (synonyms: papillary cystadenoma in lymph glands, branchiogenic adenoma, onkocytoma.) signifies a benign, connective tissue-encapsulated, histologically very characteristic tumor, made up of lymphoid tissue and characteristic tubular structures, and localized to the parotid or submaxillary regions. This form of tumor has been looked upon as exceedingly rare, and prior to 1945 only about fifty cases of such tumors had been reported — which number hardly may be taken as any reliable expression for the frequency of the tumor. Thus, Carmichael *et al.* have published 8 cases observed by themselves, and Niño found 7 adenolymphomas among seventy parotid tumors received for examination from the University Clinic of Surgery, Buenos Aires. Bilateral occurrence of this tumor has been reported only twice (Carmichael *et al.* and Niño).

In the following an account will be given of two cases of adenolymphoma (one bilateral), besides one case of a peculiar »mixed salivary gland tumor« which histologically appears related to the adenolymphoma (see below).

Case 1. (K 24443—R 43614).

The patient was a man, 65 years old, who for one year had had a tumor at the left angle of the mandible. Now it was of plumsize, somewhat protruding, rather soft, indolent, movable, non fluctuating.

On 25/1—44 exploratory puncture was performed, after which the tumor became somewhat smaller. Cytologic examination of the punctate gave no definite result, owing to necrosis.

13/4—44: Extirpation of the tumor: Under the left angle of the mandible, a little beneath the surface there was a tumor, as large as a hen's egg, smooth, quite thin-walled, non adherent that could be removed without any difficulty. It contained mushy, bluish-red and soft tissue together with small clusters of yellowish vesicles. Sections from the tumor showed large amounts of

lymphoid tissue with numerous germinal centers, besides regular tubular structures lined with regular double layer of eosinophil columnar epithelium. Some of the tubular structures were dilated so as to form small cavities, into which polyps were projecting from the wall. These polyps were covered with the same epithelium as the tubules. The larger polyps were provided with an edematous, degenerated, stroma, and it was such polyps which macroscopically appeared as clusters of vesicles.

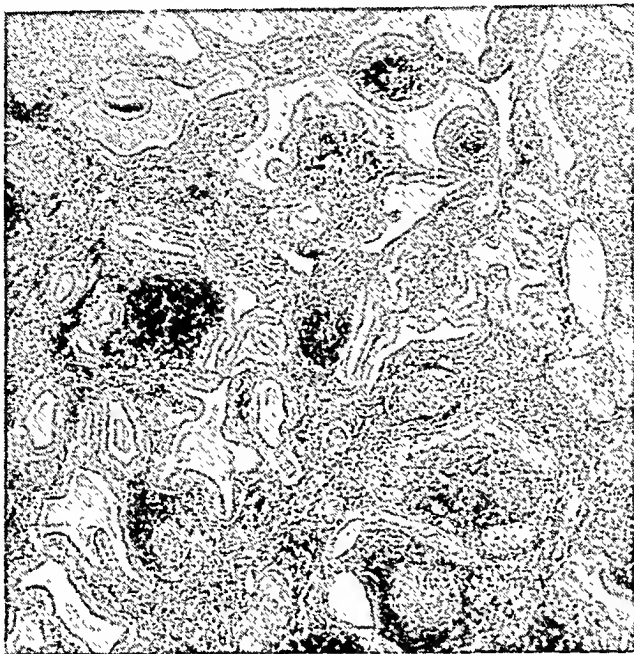


Fig. 1.

Lymphoid tissue with tubular structures. (25 \times)

Microscopic diagnosis: Adenolymphoma (Fridtjof Bang).

After three years the patient returned on account of a small ulcer that would not heal, located just over the right angle of the mandible. Besides, for some weeks he had noticed a small node under the right angle of the mandible that gave him no inconvenience. Microscopy of the ulceration showed a planocellular parakeratotic carcinoma. It was treated with radium.

Under the right angle of the mandible there was found a small firm tumor (size of a hazel-nut) which did not infiltrate the surroundings. Clinically the possibility of a metastasis could not be excluded. Under three weeks' observation it became a little smaller and softer. No sign of recurrence on the left side of the neck.

2/4—47: Extirpation of the tumor under the right angle of the mandible: The intumescence was a little larger than a nut kernel, situated just beneath the subcutaneous tissue, immediately in front of the vessels. This tumor practically slipped out. On section, the cut-surface was greyish, with a granular center; consistency not firm. Histological examination showed tumor tissue of exactly the same character as seen in the tumor removed from the opposite side three years before.

Microscopic diagnosis: (Fridtjof Bang) Adenolymphoma.

Hemoglobin: 95 %. Sedimentation rate: 15 and 8 mm. Microphotos 1 and 2.

Case 2. (K 27332).

The patient was a woman, 61 years old, in whom, 19 years before, a cyst over the right clavicle had been removed, in another hospital. That cyst had not been examined microscopically.

Now the patient seeks medical advice for a tumor situated behind the left angle of the mandible. This tumor gave no particular symptoms, but lately it had been increasing in size. Now it was as large as a plum, well isolated, soft, freely movable in relation to the skin and the underlying structures. Two explorative punctures gave no definite result (both times it was impossible in this way to obtain any tissue suitable for examination).

Only on biopsy could the diagnosis be made: adenolymphoma.

During two months' observation the intumescence decreased somewhat in size.

5/11—45: Extirpation of the tumor.

The tumor was a little over walnut-size and extended up behind the ramus mandibulae. It was removed without any difficulty. On section the cut-surface appeared somewhat necrotic, otherwise brownish red, rather edematous.

Histological examinations: Tumor tissue of the same appearance as the preceding tumors prescribed.



Fig. 2.

Regular double layer of columnar epithelium.
The inner layer with luminal nuclei. (140 ×)

Microscopic diagnosis: (Fridtjof Bang) Adenolymphoma.
Hemoglobin: 83—99 % sedimentation rate: 12—15 mm.
Reexamination, 1 year later: No sign of recurrence.

Clinical Aspects.

The tumor occurs most frequently in middle-aged and elderly men. The average age at which it is noticed is 54 years. The sex distribution is striking, the tumor being 6 times more frequent in men than in women — a proportion of which no reasonable explanation may be given at present. The site of choice is the parotid region, especially at the angle of the mandible, while in rare cases it has been encountered in the submaxillary region. Among 28 adenolymphomas gathered from the literature by Niño, 25 were located in the parotid region only three in the submaxillary region. So far, no adenolymphoma has been described without relation to the two large salivary glands. Not infrequently the growth of the tumor is peculiar, giving unjustified suspicion of malignancy: As a rule, then, the tumor keeps stationary for years, or it grows but slowly, or varies a little in size, whereafter it suddenly grows rapidly, increasing markedly in size from accumulation of secretion or inflammatory processes. In 3 of the cases reported by Carmichael and in one of the present cases there was a sudden increase in the size of the tumor.

Besides, large tumors may cause pressure atrophy of the skin, without invading the skin. The smallest tumors described have been as large as nut kernels, the largest ones of mandarine size. Most often the consistency is softer and hardly so heterogeneous as that of the mixed tumors, although they may become hard and tense from accumulation of secretion. On the whole they are submitted to treatment later than the mixed tumors; and they are more mobile, more superficial and easier to enucleate. Apart from some tenderness on sudden growth, the adenolymphoma gives but few complaints or none at all, on which account its preoperative duration often is long, averaging 6 years.

Owing to the relative scarcity of this tumor and its location in relation to the lymph nodes of the neck and to the large salivary glands, the clinical diagnosis of adenolymphoma is difficult. Thus, Carmichael (1935) states that up to that time the diagnosis had never been made preoperatively. Subsequent authors (Ramage *et al.* and Niño) emphasize the diagnostic value of aspiration biopsy; and yet the result may turn out negative on account of necrosis — just as in Niño's case of bilateral adenolymphoma and in both cases here presented. Exceptionally, adenolymphoma may occur in children, and besides being bilateral, also multicentric tumors may arise in relation to the same gland. In these cases the histological picture is often deviating from the normal, and possibly these more or less atypical adenolymphomas may contribute to an understanding of the histogenesis of the tumor, on which account they will be discussed in detail.

Macroscopic Features:

The tumor is roundish or somewhat flattened, with a lobular or finely granular surface, of variable consistency, depending on the amount of secretion and size of the cysts. On section, the cut-surface is often characteristic, reddish-grey solid areas alternating with cysts that present a mucoid content and papilliferous formations (in the writer's cases resembling a hydatidiform mole). There is a well developed, though sometimes thin, connective tissue capsule, from which septa may extend into the parenchyma. When the cyst formation is pronounced, the diagnosis may be made macroscopically; if not, the differential diagnosis from mixed tumour, metastatic tumor and tuberculosis may be difficult, depending upon the location of the tumor, capsule formation and possible presence of necrosis. (In neither of the cases here reported was there any macroscopic cyst formation, possibly because of the relatively short preoperative duration of the tumors and their relatively modest size).

Microscopic Features.

The tumor is built up of two components: lymphoid tissue and epithelial elements. Most often the lymphoid tissue is abundant, with distinct germinal centers, and sometimes with scattered plasma cells and eosinophil leukocytes. A differentiation into cortical and medullary substance has been described, and Albrecht et Arzt look upon this tissue as being undoubtedly lymph node tissue, whereas Hamperl and several investigators emphasize that the more detailed structure of the lymph node with trabeculae, lymph sinuses etc. are lacking. Here and there, areas of necrosis or hyalin masses may be seen. Embedded in the lymphatic tissue, and separated from it merely by an exceedingly thin layer of loose connective tissue, a great number of tubular structures are seen, regular in form, often distended into cysts, and sometimes presenting polyp-like projections from the wall. The glandular tubes and the polyps are lined with a characteristic regular epithelium consisting of one or two layers of high, slender, eosinophil columnar cells without cilia. The inner layer is always regular with luminal nuclei, while the outer layer — the basal layer — is less regular, and here the nuclei are rather basal. In typical cases, then, we meet with two rows of nuclei: one luminal, the other basal. The nuclei are fairly large, round or slightly oval, varying in stainability. Only a few mitotic figures are seen.

As demonstrated by Hamperl, these cells strikingly remind of a cell type occurring in normal salivary glands that Hamperl designates as »onkoocytes« (see below). Both the luminal and the basal cells contain a fine eosinophil network in the meshes of which small argentophil granules may be demonstrated. Between the network there is vacuolized protoplasm staining pink with eosin. In Mallory-stained

sections Jaffé has demonstrated intercellular secretory capillaries, which in some areas can be made out in every cell, while in other areas they are scanty. The contents of the cysts are either finely granular and slightly eosinophil or homogeneously colloid-like and markedly eosinophil. Cholesterol crystals are seen as well as desquamated epithelial cells, necrotic papillary fragments, and small concentric stratified formations reminding of corpora amylacea. Eosinophil droplets of secretion are often seen on the surface of the columnar cells.

Prognosis.

Adenolymphoma is generally looked upon as a benign tumor. Still, among the 50 odd cases reported, the tumor has been recurrent in 1 case, malignant in 3 cases.

Carmichael *et al.* have reported one case of recurrence of the tumor after its operative removal, but neither tumor showed any clinical or histological sign of malignancy.

Ramage *et al.* state that possibly this may not have been an instance of true recurrence but rather a small tumor that was overlooked at the first operation. In their own case of bilateral adenolymphoma these authors found two adjacent though separate tumors on one side. Also Gruenfeld & Jorstad have described a multicentric adenolymphoma.

Throughout the literature on adenolymphoma mention has been made of two malignant cases, one described by Ssobolew the other by Stöhr & Risak.

Ssobolew's malignant adenolymphoma came from a middle-aged man in whom a rapidly growing, non-capsulated tumor was removed from the parotid region. The author received three pieces of tissue for microscopy, one of which showed areas of adenomatous and papillomatous, less frequently solid, carcinoma, whereas the remaining two pieces showed an encapsulated adenolymphoma with very well developed branching and communicating, papilliferous formations in the cysts and with characteristic epithelial lining. The patient died a few days after the operation from some other cause.

Ssobolew admits that there was no demonstrable transition in this specimen from adenolymphoma to carcinoma. Still he maintains the diagnosis of carcinoma arising from adenolymphoma, without taking other possibilities into consideration — *e. g.*, a regional coincidence of two different tumors, one of which was an adenolymphoma, while the other may have been a metastatic carcinoma or a primary parotid carcinoma of the papillomatous type.

So it will be justified to claim that in this case the diagnosis of adenolymphoma undergoing malignancy cannot be said to have been established.

Stöhr & Risak have reported the case of a boy, 2½ years old, in

whom, within 8 weeks, there developed a stone-hard tumor, measuring $6 \times 4 \times 1\frac{1}{2}$ cm. in front of the right ear. It was difficult to remove and it seemed to have occupied the entire parotid space. No normal parotid tissue could be recognized. There was no enlargement of the regional lymph nodes. The patient was discharged as well, and no reexamination was made. Macroscopically the tumor resembled a benign adenolymphoma with cyst formation. Microscopic examination showed a well-developed capsule surrounding the sharply defined lobulated tissue. Some lobules consisted entirely of well-differentiated lymphoid tissue, while others contained structures reminding of salivary ducts. Finally, some lobules were made up of papillomatous cysts, lined with eosinophil epithelium; there were luminal columnar epithelial cells and 2—3 basal rows of cuboidal or polygonal epithelial cells with many mitotic figures. In relation to the papillomatous areas, the reactive centers of the lymphoid tissue were flattened somewhat.

Stöhr & Risak took their case to illustrate a defective anlage of the parotis with developmental disturbances, and they classified the tumor as a cystadenolymphoma of the parotis. They state that the relation of the tumor to its surroundings as well as its rapid growth were suggestive of malignancy. But the authors fail to take any definite stand on this problem beyond emphasizing the presence of many mitotic figures in the basal layers of the epithelium. No invasion by these elements into the lymphoid tissue was described — let alone invasion of the capsule or the surroundings — nor was any evidence of such invasion seen in the microphotos.

So, in this case too, no malignancy of the tumor has been proved; nor is it very likely that the authors themselves took this tumor to be malignant. Still, in the literature, it has been reckoned as an unquestionable instance of malignant adenolymphoma.

Finally, in the more recent literature, Lloyd has described an instance of an adenolymphoma-like tumor with reticulosarcomatous transformation of the stroma, while the epithelial component showed no evidence of malignant proliferation.

Histogenesis.

Concerning the origin of the adenolymphoma 2 hypotheses have been advanced. Askanazy and a few other investigators thought that this would be a matter of branchiogenic tumors, analogous with the wellknown branchiogenic fistulas and cysts encountered farther down on the neck and likewise containing lymphoid tissue and spaces lined with epithelium. The other view — that here we are dealing with aberrant salivary gland tissue — was advanced already by Albrecht & Arzl, who were the first to describe an adenolymphoma. They pointed out the similarity sometimes seen between the epithelium in the tumor and the epithelium of normal excretory ducts of the salivary

glands; furthermore — like Jaffé subsequently — they also found duct-like structures and acini in the capsule as well as in the connective tissue septa radiating from the capsule into the tumor. This view becomes of particular interest if we correlate it with the studies reported by Neisse and with Hamperl's demonstration of the »onkocytes«, also with various atypical cases of adenolymphoma.

In fetuses Neisse has found numerous scattered lymph nodes containing salivary gland tissue, corresponding to the anlage of the parotis and the submaxillary gland. To begin with, these lymph nodes are located diffusely, enclosing also salivary gland tissue, but gradually they become well defined, forming the pre-auricular lymph nodes. In nearly all newborn children such salivary gland tissue may be demonstrated within these lymph nodes, and sometimes it persists also in adults. So it seems obvious to explain the origin of the »atypical« adenolymphoma reported by Stöhr & Risak in a boy of 2½ years in keeping with the studies reported by Neisse. Further the characteristic thorough mixture of glandular structures and lymphoid tissues in typical adenolymphoma may undoubtedly be explained in this way too.

Subsequently Hamperl called attention to the »onkocyte« (*i.e.*, »Swollen cells«), which also normally differentiate in the excretory ducts of the salivary glands with increasing age, and which strikingly resemble the characteristic epithelial cells in the adenolymphomas. After the age of seventy these cells may be found almost regularly, while they nearly almost are absent before the age of twenty. Hamperl also demonstrated focal »onkocytic« hyperplasia in elderly patients as well as transitions to adenomatous structures combined with lymphoid tissues. Onkocytes have been demonstrated also in the pancreas and parathyroid (Hamperl), in the pituitary (Löffler and others), even in the glandular epithelium of the Falloppian canal (Hamperl), and recently in the mucus membrane of the nose and larynx (Nohteri), but no adenolymphoma has yet been described outside the parotid and submaxillary regions. On the other hand, onkocytes may enter into the make-up of benign tumors of various organs — and exceptionally in malignant tumors too as, for instance, in the malignant adenoma of the thyroid described by Langhans. Nohteri has described an internal laryngeal cyst lined with onkocytes, but lymphoid tissue was not seen, and it was not a branchiogenic cyst. In addition, in the nasal and laryngeal mucus membrane in old people, Nohteri has now and then observed a tendency to adenomatous proliferation. He thinks that the onkocytes are cells that loose their original functional capacity and then undergo redifferentiation. Owing to the capacity of these cells for proliferation it seems hardly likely to be a degenerative phenomenon; indeed, no relation has been found between senile vascular changes and the occurrence of onkocytes.

Perhaps the case reported by Gruenfeld & Jorstad represents a transitional form from Hamperl's focal onkocytic hyperplasia to clear-

cut adenolymphoma. These 2 authors found an encapsulated tumor, which again contained several small tumors which were separated mutually by fibrous connective tissue or by areas of normal salivary gland parenchyma. From the description and microphoto these individual small tumors appear to be closely related to the typical adenolymphoma, and by serial sectioning the authors were able to demonstrate the starting point of groups of tumor cells in an excretory duct.

This conception of the histogenesis of the adenolymphoma finds support in the following case of »mixed salivary gland tumor« from the Histopathological Laboratory of the Radium Station:

Case 3. (K 32251).

The patient was a woman, 74 years old, who for the last 8—10 years had had a small tumor of the prolabium on the left side of the upper lip. Now it had attained the size of a pigeon egg; it was firm, indolent and well defined. The tumor was easily removed in toto. The surface was slightly nodular, the cut-surface white in color. Histological examination showed a well defined tumor tissue which in some areas was built up quite regularly of close-packed tubular canals lined with eosinophil columnar epithelium of the type seen in adenolymphoma of the salivary glands (onkocytes). But there was no lymphatic tissue in the stroma. In another part of the tumor the structure was more irregular, the cells in the glandular tubes appearing here in several layers, being poorly differentiated, and often forming small solid heaps in a stroma



Fig. 3.
Mixed tumor with tubular structures
and onkocytes. (25 ×)

which — like the stroma in mixed tumors of the salivary glands — is myxomatous in some areas, cartilaginous in others.

Microscopic diagnosis (Fridtjof Bang): Mixed tumor with onkocytes. (Microphotos 3,4).



Fig. 4.

Transition from onkocytes to the smaller cells
in the mixed tumor. (140 ×)

This case, in which a part of the tumor shows a structure greatly reminding of the epithelial component of the adenolymphoma (though without the characteristic arrangement of the nuclei), while another part shows a typical »mixed salivary gland tumor« speaks in favor of the view that the epithelial elements of adenolymphoma develop from special salivary gland cells, while it also lends support to the theory that the so-called mixed salivary gland tumors are to be looked upon as adenomas with secondary changes.

Histologically quite typical multicentric (or bilateral) instances of adenolymphoma have been reported by Niño and by Ramage *et al.*, to which now may be added one of the cases here presented.

It is still an unsettled question whether the lymphatic tissue takes any active part in the tumor formation. A certain relation between the proliferation of the onkocytes and the occurrence of the active centers in the lymphoid tissue has been taken to signify a tumor activity of this tissue. But, as pointed out by Jaffé, such reactive centers will develop at any time when increased absorption in the lymphoid tissue is required. So this circumstance may conceivably offer an explanation of the frequent, though not constant, parallelism between the onkocytes and the lymphatic tissue.

The various studies and considerations cited here, I think, are probably to be interpreted to this effect: adenolymphomas are benign tumors, arising from embryonal anlagen of salivary gland tissue, in particular from onkocytes in the excretory ducts of these anlagen. Likewise, the lymphoid stroma is made up of embryonal remnants from these anlagen that have undergone hypertrophy in the coordination with the proliferation of the onkocytes.

The *treatment* of these tumors consists in operative removal. X-ray treatment may give a considerable reduction in the size of the tumor, but not complete disappearance. As a possible explanation of the effect of radiotherapy, Ramage *et al.* mention a decrease in the secretion, but it seems reasonable also to point out that lymphoid tissue generally is very sensitive to irradiation.

Summary.

Two cases of typical adenolymphoma are reported, one of them bilateral. In both cases the diagnosis was uncertain until histologic examination had been made. Aspiration biopsy was performed without success, owing to necrosis.

On the basis of previous communications, the writer gives a survey of the clinical and pathological aspects of adenolymphoma, pointing out that so far no case has been described of unquestionable transformation of adenolymphoma into carcinoma, whereas an instance of reticulosarcoma in an adenolymphoma has been reported. The histogenesis of the tumor is discussed and, for elucidation of this question, mention is made of a peculiar mixed salivary gland tumor that appears to be related to the adenolymphoma.

An attempt is made briefly to offer an explanation of the effect of X-ray treatment in cases of this kind.

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SOME ANIMAL EXPERIMENTS ON THE INFLUENCE OF SANOCRY SIN ON HYPERSENSITIVENESS

By *J. Ørskov & E. Krag Andersen.*

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There is some justification for saying even now that we do not know much about the mode of action of sanocrysin*) in the human and animal organisms.

However, it seems relatively certain that there is a therapeutic effect, one that is difficult to reject, on some diseases, perhaps especially on some cases of chronic rheumatoid arthritis. The idea that this affection might have some connection with certain conditions of hypersensitiveness started these experiments on the possible effect of sanocrysin on the course of anaphylactic shock in guinea-pigs.

It quickly became apparent that once hypersensitiveness to horse serum had set in, it was difficult to find any distinct effect from sanocrysin, whereas there seemed to be no doubt of some degree of influence on the quality of the hypersensitiveness engendered after a pre-treatment for a shorter or longer period with sanocrysin prior to sensitization. This can be demonstrated clearly by means of a suitable adaptation of the experimental conditions, as shown for instance in Table I.

Only one out of twelve animals treated died, despite primary reactions in the majority of them, whereas nine of the twelve untreated control animals died from the shock.

In this connection it would be interesting to see whether sanocrysin treatment has any influence on the tuberculin reaction in tuberculous animals.

Out of curiosity we made a small experiment in order to see whether sanocrysin has any effect on the formation of antibodies in rabbits.

*) We take this opportunity of thanking the Ferrosau Company for kindly supplying the sanocrysin necessary for the experiments.

Table I.

Twelve guinea-pigs injected intravenously (ear vein) on Dec. 30th with 0.25 cc., Jan. 2nd 0.25 cc., Jan. 5th 0.25 cc., Jan. 8th 0.25 cc., per kg. bodyweight; on Jan. 12th the animals were sensitized with 0.5 horse serum 1—10 intraperitoneally; on Jan. 27th 0.2 c.c.m. horse serum intravenously.

	Weight Dec. 29.	Weight Jan. 10.	Reaction Jan. 27. mins.	
5436	500	470	2.5	survives
5437	510	490	3	survives
5438	500	530	3	survives
5439	500	460	2.5	survives
5440	485	500	2	survives
5441	470	350	2.5	survives
5442	490	470	7	survives
5443	500	470	1.5	dies
5444	490	500	2	survives
5445	490	490	—	survives
5446	500	450	1.5	survives
5447	500	470	1.5	survives

Twelve control guinea-pigs sensitized Jan. 12th with 0.5 horse serum 1—10 intraperitoneally; Jan. 27th the animals injected with 0.2 c.c.m. horse serum intravenously.

4349	—	—	4	dies
5449	520	530	2	dies
5450	480	520	4	dies
5451	495	490	3	survives
5452	480	480	3	dies
5453	510	530	1	dies
5454	500	530	1.5	dies
5455	495	500	—	survives
5456	480	490	1.5	dies
5457	500	530	2	dies
5458	500	520	4	dies
5459	490	490	1	survives

Three rabbits weighing 3 kg. were injected on Dec. 30th with 0.5 cc., on Jan. 2nd 0.5 cc., Jan. 5th 1 cc., Jan. 8th 1 cc., Jan. 10th 2 cc. and Jan. 13th 2 cc. of sanocrysin per kg., intravenously.

On Jan. 15th we injected intravenously 0.1 c.c.m. 24-hours' old paratyphoid B broth culture killed in formal, and simultaneously three rabbits of the same weight were injected intravenously with the same dose of paratyphoid B culture.

The sanocrysin-treated animals lost a little weight during the treatment but otherwise presented no morbid symptoms. A few hours after the injection all three animals pre-treated with sanocrysin to our great surprise became moribund; two died within 24 hours, but the third survived after several days of severe morbidity. As was expected, the controls gave no reaction whatever in the way of morbid symptoms to the injection.

For the rest the formation of antibody as regards H-agglutinin proceeded in the sanocrysin-treated animal as in the controls. There can be no doubt but that sanocrysin brought about a most conspicuous reduction in the resistance of the animals to the intravenous injection of bacterium culture.

Summary:

In animal experiments under suitable conditions sanocrysin may affect the course of the anaphylactic shock. In animal experiments sanocrysin may reduce the resistance of an organism to toxic effect.

POLIOMYELITIS IN MICE*)

By J. Ørskov & Else Krag Andersen.

(Received for publication June 2nd, 1948.)

Our principal reason for embarking upon experimental work on mouse poliomyelitis is because mice have the advantage of being cheaper to do with than apes and presumably are just as suitable for assisting in throwing light upon questions both theoretical and practical concerning poliomyelitis.

Earlier virus research (1) imbued us with the desire to try by means of similar experiments to learn something about the mechanism of infection in both normal animals and animals immunized in various ways; more particularly, the possibility of being able within our limits to work on natural routes of infection was tempting. Even in his first works *Theiler* (2—3) refers to the distinctly greater susceptibility of young animals to Polio virus; to us there was nothing surprising in this, but to our knowledge no one else has yet grappled with this possibility systematically. *Gard* in Sweden obligingly sent us the so-called *Theiler F.A.strain*, with which we have worked a good deal. As in other laboratories, we quickly found that most of the animals in our stock were enteroinfected *Olitsky* (4), but that it caused no sickness among them except for the extremely few instances of paralysis we have seen now and then among the tens of thousands of mice used annually at the Serum Institute. We have isolated many of these intestinal strains, and on one occasion found one which in virulence was comparable with the *F.A.strain*. On testing most of these spontaneous intestinal strains in feeding experiments with young animals we find that it is only within the first 14 post-natal days that some of them — the number varies — are attacked by Poliomyelitis, in most cases after a relatively long period of incubation. The resistance of the different litters varies considerably; in some a relatively high percentage are attacked, in others none at all, which experimentally is a drawback though a matter of great interest. We

*) Read in abbreviation before the Fourth International Congress for Microbiology, Copenhagen, July, 1947.

are trying to rear a virus-free stock material, and hope to succeed, so that something more may be learned about the behaviour of these relatively low-virulent strains with respect to infection and immunity. The F.A. strain is excellent for the purpose, for it possesses a relatively constant pathogenicity administered by the mouth to very young mice until they are about 14 days old. In some tests it has displayed a very variable power of infecting still older mice by the mouth, but this power is not constant, as already stated.

Our method of carrying out the peroral infection is to hold the mouse by the neck skin with soft forceps and with a Pasteur pipette place a small drop on the lips; if its tail is rolled gently between two fingers the mouse opens its mouth and drinks the drop. Undoubtedly some gets up inside the nose now and then, as we ascertained by mixing the virus suspension with Indian ink; but this is relatively rare if the operation is performed carefully, and, moreover, nasal infection is presumably of no great importance as far as the mechanism of infection is concerned.

With these young animals the infectivity is very high. Dilutions right up to 1:100,000 give a high percentage of takes. There seems to be but little difference in the resistance of the various litters, so that there can be no question of any great immunity inherited from the mouse mothers infected with low-virulent Theiler virus in the intestine. As a matter of fact, at an early stage of the work we thought it strange that young mice, susceptible as they are, do not become infected much more often in their first weeks, especially as it is not very rare to find intestinal strains of a relatively high virulence, and as even the low-virulent strains in a number of instances are capable of causing paralysis in suckling young after peroral administration. In order to delve deeper into this we experimented with smearing the mother's teats with virus suspension, at the same time putting a collar on the mother to prevent her from licking her teats clean. Nothing happened to the young, in spite of repeated applications. Accordingly we thought that perhaps the admixture of milk might cause such a change in the consistence of the food that the risk of infection was reduced. Being unable to milk the mothers we took fresh gastric contents from the young and mixed them with virus. Again the result was hardly any infectivity. There was no destruction of virus by the stomach contents; after centrifuging, the supernatant fluid was highly infectious; the same thing happened when we added normal, undiluted serum: the infectivity was reduced. Believing that the nearest explanation must lie in the consistence of the suspension fluid, we tried feeding the animals with undiluted infected brain — or diluted only with an equal part of water. The result was practically the same: very few animals were infected, and as we were able to find virus in the intestine of the animals only a couple of hours after administration, there can scarcely

be any doubt that as far as this virus is concerned, the form in which it is ingested is of decisive importance; at the same time, all the signs seem to indicate that the infection occurring in conjunction with peroral administration must in the majority of cases take place in the bucco-pharyngeal section of the alimentary canal.

We may say at once that the rules applying to this virus strain do not apply to either Jungeblut S. K. or M. M. strains, both of which are highly virulent when given undiluted to both young and old animals, whatever may be the explanation.

For the purpose of ascertaining in what localities the virus can be found in orally infected animals we infected some litters of young, all 5—6 days old. They were killed at increasing intervals, the first two hours after administration, and various localities of interest to us in connection with the infection mechanism were removed and injected intracerebrally into fresh animals. Although there was no reason for expecting to find virus ubiquitously from these apparently highly neurotropic forms of virus, an extraneural demonstration would have been of interest on immunological grounds. Very interesting in this connection are the relative recent findings of *Dorothy Horstmann* and others, (5) experimenting with young rhesus monkeys infected orally with Polio virus. In one of these animals they found lymph nodes, spleen, heart and adrenals infected. It is not often that Polio virus is observed outside the nervous system — except in the alimentary tract; but still there are some cases, and they may perhaps be more

YOUNG MICE (5 DAYS OLD) F.A. PER OS.																														
		SUBMAXILL. GL.				ARL. THYROID GL.				MESENT. GL.				LIVER				SPLEEN				BLOOD				BRAIN				
		1/10		1/100		1/10		1/100		1/10		1/100		1/10		1/100		1/10		1/100		1/10		1/100		1/10		1/100		
		1		2		1		2		1		2		1		2		1		2		1		2		1		2		
No																														
4 h. after feeding	19/4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
10	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
24	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
48	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
2 d.	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3	—	15	16	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
4	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
6	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
9	—	8	7	9	14	11	11	11	0	4	0	0	11	7	9	9	0	16	0	0	0	0	0	0	0	0	0	7	4	6
	35/3 ill.																													

YOUNG MICE F.A. PER OS																													
		SUBMAXILL. GL.				INTESTINES				MESENT. GL.				LIVER				SPLEEN				BLOOD				BRAIN			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
7	d. old Mice																												
9	- after feeding ill	0	11	9	8					0	0	5	0	7	7	8	9	10	10	10	0	0	0	0	0	0	0	0	0
		8	8	6	7	0	0	9	0	13	0	0	0	5	8	7	15	17	10	10	10	0	0	0	0	0	0	0	0
		6	7	10	6	0	11	0	8	0	0	0	0	13	8	11	7	8	0	22	10	0	14	0	0	0	3	3	3
18	d. old Mice																												
13	- after feeding ill and paralysed.	0	0	0	0					0	0	0	0	0	11	0	12	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	4	0	11	0	0	0	0	0	0	0	0	0	0	0	0	9	10	8	0	9	10	8

Table I.

Dilutions of $\frac{1}{10}$ and $\frac{1}{100}$ were made from every sample, and two mice, Nos. 1 and 2, were injected intracerebrally with each dilution.

frequent in future. It would also be interesting if one could find a systematic diffusion via the lymphatics such as that of practically all bacterial infections and several virus infections, for example vaccinia virus and fowl plague virus. (1. & 6). As we see from Table 1, there is nothing really systematic about the finds; but it is clear that at a very early stage — long before the central nervous system is demonstrably infected — we may find virus in blood, liver and glands, and that during the first hours and days the blood is very frequently infected, whereas the brain does not become a seat of infection until the second day at the earliest. The longer the infection progresses the more often is the brain infected and the more positive finds do we in most cases make elsewhere, while simultaneously the infection in the blood becomes more difficult to demonstrate, though we have made no effort to ascertain whether the latter is or is not due to an actively occurring relative immunity.

From the experiments it seems possible to draw the conclusion that besides the infection of the nervous tissue, a relative diffusion takes place in orally infected animals, and that apparently this diffusion does not necessarily take place exclusively via the nerve paths.

F. A. 0,25 1:100 I.V.

	BLOOD															LIVER														
	1:10					1:100					1:1000					1:10					1:100					1:1000				
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
24. AFTER I.V. INJ.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5"	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
24"	-	-	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

S. K. 0,25 1:100 I.V.

	BLOOD															LIVER															
	1:10					1:100					1:1000					1:10					1:100					1:1000					
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	
24. AFTER I.V. INJ.	1	2	3	3	3	3	3	3	2	3	3	0	3	3	2	2	2	3	2	3	5	2	4	3	3	3	4	3	6	3	3
5"	—	—	—	3	4	4	5	4	6	6	8	5	0	0	6	0	0	4	0	0	5	3	3	3	5	4	4	0	4	5	3
24"	—	—	—	4	2	1	4	4	4	3	3	3	4	3	5	4	0	4	0	4	4	3	3	2	3	3	4	4	4	5	3

Table II.

Theiler F. A. virus and Jungeblut S. K. tested for elimination after intravenous injection. Intracerebral test of the various suspensions on six mice.

A small experiment shows that F. A. virus is bound inter alia by the liver after intravenous injection. At the same time we see how quickly the blood of the adult animal is cleared. We also see that already after 24 hours there is a distinct decrease of the quantity in the liver, showing, as was anticipated, that there is no perceptible growth in that organ. With Jungeblut S. K. virus (7 & 8) (kindly sent us by Dr. Claus Jungeblut) this is more difficult to demonstrate.

At an early stage in our experiments we saw that mice which had survived F. A. paralysis could have immune offspring. In experiments in actively immunizing mothers before gestation, aiming at giving the offspring a passive immunity, the results we obtained were very variable. As a rule we prepared the vaccine from mouse brain taken at the culmination of the disease, diluted to 1:100 and killed with 0.1 % formol. In some of the experiments we got fine results, in others not so good. The most positive results were obtained by inoculating with killed and then living material, both intraperitoneally. In Table III we have an example, where all the young survived peroral infection whereas all the controls died.

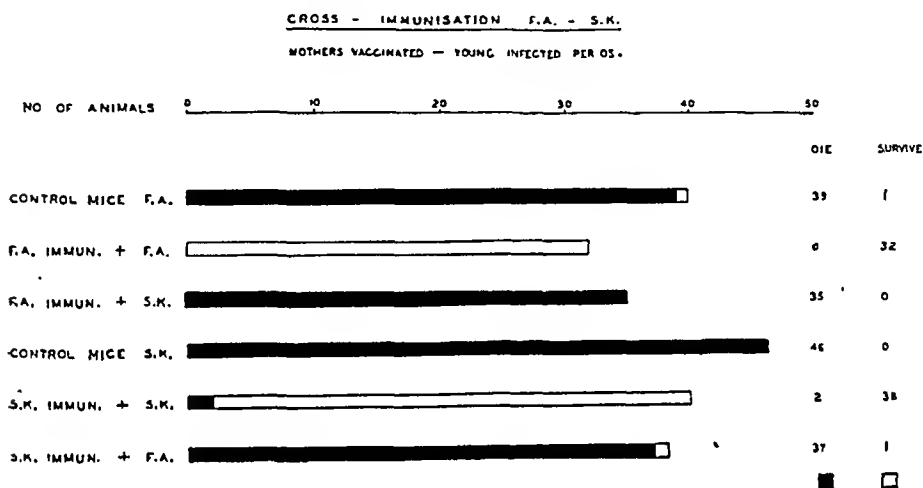
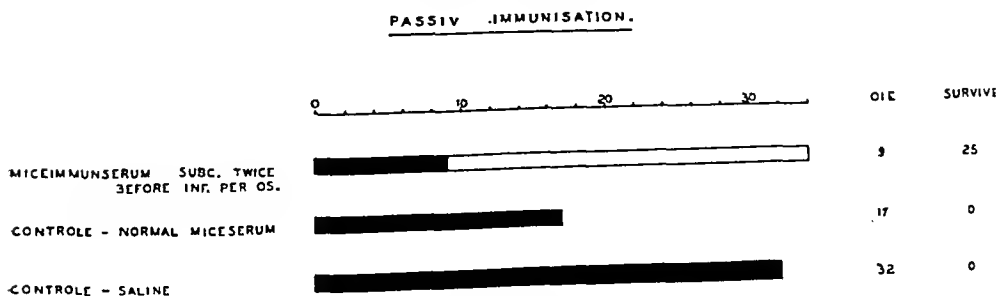


Table III.

Mothers vaccinated first with killed, then with live vaccine intraperitoneally. Only one of the young control mice infected with F. A. survives, while no Jungeblut S. K. control mice survive.

In other instances it seems that very little is required for the mother to obtain an immunity sufficient to give the young a considerable measure of protection; for instance, having suckled a litter of infected young was in some cases enough to give the next litter fair passive pro-

Table IV.
Passive immunization.

tection. We also found that it is relatively easy to protect young against peroral infection with serum from immunized animals.

Table IV provides an example. Six litters of young at three and five days old were injected with about 0.2 c. c. mouse immune serum subcutaneously. Of these 34 young, 25 survived whereas all the 17 controls died. Saline and normal mouse serum had no protective effect. It was interesting to see that the nine young that died had a considerably protracted disease period, averaging 22 days, i. e. about twice the usual length. On the other hand, we were never successful in giving young a passive protection by inoculating the mother repeatedly during gestation with large amounts of mouse immune serum.

We have also made numerous experiments with Jungeblut's S. K. strain, which is also infectious perorally for older animals. What is the mechanism of infection here? We find the same irregular diffusion

MICE (18 g.) S. K. PER OS.

	SUBMAXILL. GL.								LIVER								BLOOD								INTESTINES			
	1:10				1:100				1:10				1:100				1:10				1:100				1	2	3	4
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4								
2 h. after feeding	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	
5 - - -	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
10 - - -	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	34	
24 - - -	3	2	3	2	3	2	4	4	0	0	0	0	0	3	3	0	3	0	0	3	0	3	4	4				

	SUBMAXILL. GL.				ING. + AXIL				MESENT. GL.				LIVER				SPLEEN				BLOOD				BRAIN			
	1:10		1:100		1:10		1:100		1:10		1:100		1:10		1:100		1:10		1:100		1:10		1:100					
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2				
1 d. after feeding healthy	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	7	0	0	0	0	0	0	0	0	0	0	0	
2. - - -	5	7	0	6	7	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
3. - - -	7	9	8	8	5	0	0	8	0	0	0	0	0	0	0	0	5	0	6	11	0	0	0	0	0	0	0	
4. - - - typ. ill.	5	4	5	9	0	0	0	0	5	5	4	0	4	5	7	7	4	7	7	8	0	8	0	0	2	2	3	
5. - - -	4	4	5	5	4	4	3	3	5	5	4	5	5	5	0	0	6	4	4	5	11	0	0	0	3	3	3	

Table V.
Jungeblut S. K. per os to adult mice.

but in these relatively old animals no demonstrable, very early diffusion. Interesting is a case 3 days after infection, when several localities are infected but not the brain yet, demonstrably at any rate. It is worth mentioning here that as in many other experiments, the strains which we secured from the intestine are of the low-virulent Theiler type.

With the S. K. strain we were also successful in producing immunity in the young by giving killed vaccine to the mother, but here again the killed vaccine + living acts more certainly than killed alone. Here it turned out to be surprisingly easier to immunize the mother against infection with living virus, e. g. intraperitoneally, than it was to induce passive immunity among the young; for instance, not one of 40 mothers died after three intraperitoneal injections of formol-killed

vaccine followed by injection of an otherwise fatal dose of living virus. We have made no attempt to ascertain whether interference played any role in this. Such a treatment with killed vaccine is not always sufficient to give the offspring protection.

<u>MICE VACCINATED WITH FORMOL - KILLED S.K. I.P.</u>																																				
<u>YOUNG INFECTED PER OS. S.K. 10⁵</u>																																				
	1 st LITTER									2 st LITTER									3 rd LITTER									4 th LITTER								
NO. OF ANIMALS	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
MOTHER NO.1	■■■■■■■■■									■■■■■■■■■									■■■■■■■■■									■■■■■■■■■								
CONTROL	■■■■■■■■■									■■■■■■■■■									■■■■■■■■■									■■■■■■■■■								
MOTHER NO.2	■■■■■■■■■									■■■■■■■■■									■■■■■■■■■									■■■■■■■■■								
CONTROL	■■■■■■■■■									■■■■■■■■■									■■■■■■■■■									■■■■■■■■■								
MOTHER NO.3	■■■■■■■■■									■■■■■■■■■									■■■■■■■■■									■■■■■■■■■								
CONTROL	■■■■■■■■■									■■■■■■■■■									■■■■■■■■■									■■■■■■■■■								

Table VI.

Mothers vaccinated with killed Jungeblut S. K. virus intraperitoneally; their young in consecutive litters tested for resistance.

Table VI shows that the ability of mothers treated in this manner with killed vaccine to give the offspring passive protection is very variable. In the control litters all died except one. Mother No. 1's young all died in the first litter after the inoculation, and the experience of nursing the sick young has no influence, as the next three litters all died too. Mothers No. 2 and 3 were unable to give the young of the first litter immunity enough to survive the infection, whereas everything went well with the next three litters. In this case it would thus seem that the encounter with the living virus governs the quality of the antibody in the mother's blood.

In conclusion, a cross-immunity test between F. A. Theiler and S. K. Jungeblut (Table III). As usual, offspring of mothers inoculated with F. A. and S. K. respectively were tested against both F. A. and S. K. We see from the experiment that the F. A. and S. K. vaccines have no mutually protective power, whereas they afford good protection against the homologous strains.

Finally, we made an experiment in order to ascertain whether there was any cross-immunity between Yale S. K. (kindly sent us by Professor John R. Paul) and the mouse-adapted S. K. Jungeblut. On account of the inability of the Yale strain to infect young mice orally we were unable to make the tests in the usual manner, and therefore the experiment had to be restricted to a demonstration of whether mothers very actively vaccinated with Yale S. K. could give their young relative

protection against the other mouse-adapted strain. The experiment was performed with 9 mothers and their young, and the mothers were vaccinated prior to gestation with very large quantities of killed and living culture intraperitoneally. For controls we had a similar number of litters whose mothers had been vaccinated with killed + living Jungeblut S. K. virus. Except for one animal, all these control young survived, whereas 57 of 58 young which were tested for immunity against Jungeblut S. K. per os, died. Thus by this method there was not the slightest demonstrable immunity.

Summary.

The low-virulent faeces strains that are commonest in mice give a very irregular percentage of takes when mouse young from arbitrarily selected litters are infected perorally in their first 14 post-natal days. Most of the litters in our stock were fully resistant, whereas only few showed a relatively high percentage of takes. Consequently, these virus strains were unsuitable for immunity investigations under our laboratory conditions.

Theiler's more aggressive F. A. strain, however, gave a very high percentage of takes, even in very low concentrations administered orally within the first 14 post-natal days. The consistence of the suspension medium seems to be a very important factor in the ability of this virus to register a take when administered per os; for example, suspension in milk reduced the percentage very considerably, although the virus was easily demonstrated in the alimentary canal of the animals after feeding; this perhaps is of significance in relation to the mechanism of infection.

From these experiments we believe it justifiable to conclude that where this strain is concerned the great majority of infections must take place via the upper parts of the alimentary canal.

Experiments on the mechanism of infection showed that an irregular diffusion of the virus takes place early in the organism, before the central nervous system is demonstrably infected. They seem to show that growth must take place, presumably in nerve tissue, outside of the central nervous system.

Against these viruses the liver and spleen play a similar blood-purifying role as against bacteria and many other forms of virus. No demonstrable growth of virus takes place in these organs.

By means of immunizing the mothers prior to gestation it is relatively easy to give the young substantial passive immunity. When pre-treated with serum from immunized mothers the young can be made relatively resistant to peroral infection. In most cases the animals present no symptoms of infection and those that are attacked have a very protracted sickness period.

For Jungeblut's mouse-adapted S. K. strain the infection mechanism proved to be similar to that found for Theiler F. A. Here too it was relatively easy to give young a passive resistance to peroral infection by vaccinating the mothers before gestation, the most effective being by means of a combination of killed and living vaccine. It was found from some experiments that killed vaccine alone was insufficient in many instances, but that this together with the experience of nursing an infected litter of young enabled the mother to give her next litter complete passive resistance.

With the experimental technique employed we were unable to find any cross-immunity between Theiler F. A. and the Jungeblut S. K. strain, nor could we with the Yale S. K. strain demonstrate any protection against Jungeblut's S. K. strain.

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PATHOGENIC-APATHOGENIC TRANSFORMATION OF SALMONELLA TYPHIMURIUM

II. INDUCED CHANGE OF RESISTANCE TO COMPLEMENT (CONTINUED).

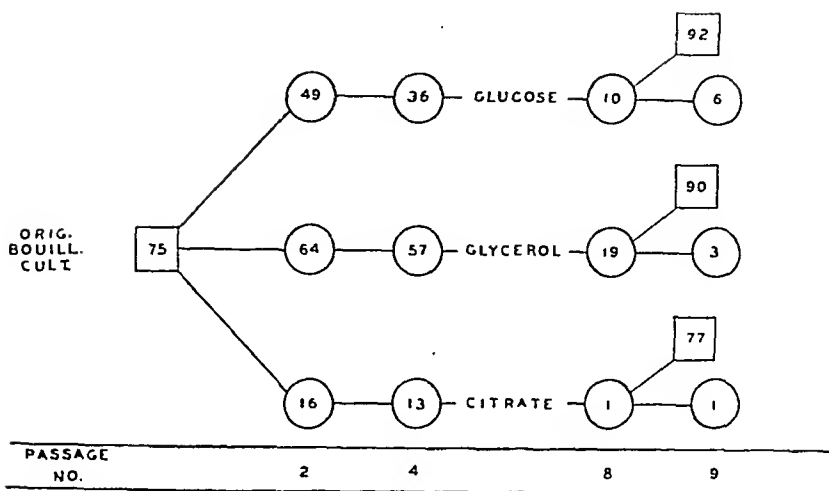
By O. Maaloe.

(Received for publication June 3rd, 1948.)

In a recent paper on this subject (1) the writer pointed out the relation between pathogenicity and resistance to the antibacterial action of complement. It was furthermore shown how growth in *diluted bouillon* resulted in the formation of bacteria with low resistance as compared to that of bacteria grown in full bouillon or in diluted bouillon to which was added small amounts of one of several simple, N-free compounds. It was emphasised that in this way the resistance was only lowered temporarily as the bacteria on transfer to a richer medium regained their normal, high resistance. — In this paper experiments with bacteria grown in chemically defined media will be dealt with.

Using very simple media of the following composition: n/15 phosphate buffer + MgSO_4 (0.01 %) + $(\text{NH}_4)_2\text{SO}_4$ (0.1—0.4 %) + Na-citrate (1—2 %), and inoculating with about 10^3 bacteria/ml taken from a fresh bouillon culture bacteria with very low resistance were immediately obtained. The same low resistances resulted when glucose (0.1 %) or glycerol (1.5 %) was used as carbon source; only in these cases the fall in resistance did not occur as rapidly as in the citrate medium. Table I shows the results of an experiment with serial cultures in the three media mentioned and it is indicated how rapidly and completely the normal resistance is regained on transfer to full bouillon. As will be shown later a more prompt effect is found in the glucose and glycerol media when appropriate concentrations of the C-compounds are chosen; for experimental details see the text accompanying Table I.

Table I.
Serial Cultures in Synthetic Media.



The numbers in the figure indicate the survival percentages of bacteria treated for 30' with fresh, normal human serum 50 %; (the technique employed has been described earlier by the writer (3)). Squares indicate cultures in full bouillon. Circles represent cultures in synthetic media with the carbon sources indicated in the figure; the exact compositions of these media are given in the text. — Here, as in all following experiments, 5 ml. of the medium are measured off in a thoroughly cleansed tube, 18 mm in diameter, a cotton stopper is inserted and the tube heated on a water bath at 100° for 15–20 min. — After cooling down the primary inoculation is made with 1–2000 bacteria/ml. from a freshly diluted culture. Secondary transfers are made directly with a platinum loop, the volume transferred being about 0.003 ml.

The carbon compounds used in this experiment all belong to the group of substances which, if added to diluted bouillon, prevent fall of resistance (cf. (1) Table IV). This fact lead to the hypothesis, forwarded by the writer (2), that in addition to one of these effective carbon compounds some substance contained in the bouillon might be necessary for the development of full resistance. This seemed the more likely as it was found that small amounts of the watery muscle extract contained in our bouillon, when added to the citrate medium, completed this in such a way that bacteria with relatively high resistances were formed. — Attempts at an isolation of the supposed factor in the muscle extract did, however, not give promising results: — some active material was found to pass through a cellophane membrane and, nevertheless, after 48 hours dialysis against running tap water, considerable amounts of active material were still found inside the bag. Thorough treatment of the extract with active charcoal removed 80 % of its N-content but left its activity practically undiminished; neither was the activity of the extract seriously impaired by heating to 100° for 12 hours at pH 1, 7 or 13. Treatment with surplus or $\text{Ba}(\text{OH})_2$ and subsequent neutralization with CO_2 did, however, remove most of the activity.

As the experiments with muscle extract pointed to some relatively simple substance as the active factor, the effects of addition of pure

amino acids to the citrate medium were eventually investigated. It was found that with glycine, aspartic or glutamic acid as N-sources instead of ammonia (and with relatively low concentrations of citrate in the media; see later in this paper) bacteria with high resistances were formed. Furthermore it was found that even after 9 passages in an amino acid medium without ammonia no drop in resistance could be detected. Finally, it was seen that in Souton's medium low resistances were only obtained when the citric acid, according to the perscription, was neutralized with ammonia; if Na-citrate was used in the place of citric acid + ammonia no drop in resistance occurred.

The experiments just cited showed clearly that *when ammonia is introduced into the growth medium the resistance of the bacteria formed is lowered*. This effect of ammonia, alone or together with muscle extract or glutamic acid, is illustrated in Tables II and III.

Table II.

Composition of Media and Resistances of Bacteria grown for 24 Hours in the Media.

Medium No.	1	2	3	4	5	6	7
Na-Citrate	0.2 %	0.2 %	0.2 %	0.2 %	0.2 %	0.2 %	0.2 %
(NH ₄) ₂ SO ₄	0.4 %	0.4 %	0.4 %	—	—	—	0.4 %
Na ₂ SO ₄	—	—	—	0.4 %	0.4 %	0.4 %	—
Carbon treated muscle extract	50 %	10 %	2 %	50 %	10 %	2 %	—
Survival % of bacteria	78 %	64 %	44 %	113 %	109 %	104 %	35 %
Viable / count 10 ⁷ /ml.	40.0	10.8	7.2	20.8	8.4	5.5	2.4

A watery, boiled extract of minced beef muscle is used. Before use in this experiment the extract has been shaken vigorously with 10 % (by weight) of active charcoal and filtered.

Table II shows how the media 4—6 in which the muscle extract constitutes the only N-source give bacteria with very high resistances; actually the resistance is so high that the few bacterial divisions which may occur during the 30 minutes treatment with fresh serum completely obliterates the bactericidal effect; hence the odd »survival percentages« above 100. — On the other hand, the media 1—3, which all contain 0.4 % (NH₄)₂SO₄ in addition to the extract, give bacteria with resistances that are lower than in media 4—6 and which diminish as the ratio organic-N/inorganic-N diminishes. With the lowest concentration of extract (medium 3) the resistance of the bacteria is nearly as low as in the control medium (7) without organic N. (It should be

mentioned here that when the control value, in this experiment is found to be as high as 35 % the reason is that the concentration of citrate is relatively low; cf. Table IV and the corresponding text.)

Table III
Composition of Media and Resistances of Bacteria grown for 24 Hours in the Media.

Medium No.	1	2	3	4	5	6
Na-Citrate	0.45 %	0.45 %	0.45 %	0.45 %	0.45 %	0.45 %
(NH ₄) ₂ SO ₄	1 %	1 %	1 %	1 %	1 %	—
Glutamic acid..	0.36 %	0.036 %	0.009 %	0.0023 %	—	0.36 %
Survival % of bacteria	30 %	62 %	32 %	8 %	14 %	95 %
Viable count / 10 ⁷ /ml.	112	118	113	70.2	64.0	6.22

Table III shows that very similar effects may be obtained with glutamic acid as organic N-source. Moreover it is seen that in the media containing ammonia + amino acid, the effect of the latter was greatest at a concentration of about 0.04 % and diminished on both sides of this optimum.

As mentioned above, it is not only the introduction of the inorganic N-source (ammonia) that causes resistance to drop. If bacteria with really low resistances are wanted it is of even greater importance that a suitable concentration of the C-compound is chosen. The greater part of the experiments illustrating the importance of the concentration of the C-compound have been made with Na-citrate, and it has been found that with a concentration of about 2 % a constant and great fall in resistance occurs in the first passage. When the citrate concentration is lowered the fall is less pronounced, and with low concentrations (0.2—0.05 %) resistances varying between about 15 and 50 % are found. Corresponding results have been obtained with glucose, glycerol, Na-succinate and Na-lactate as C-sources; in these cases concentrations of 2, 6, 3 and 2 % respectively have given primary cultures with low resistances, while with concentrations of the order of 0.1—0.5 % considerably higher resistances have been found. Based on a long series of such experiments the following general rule can be formulated: *only with concentrations of about 2 % or more of the C-compound can really low resistances be expected in the first passage (i. e. < 10 % surviving bacteria; see Table IV).*

The experiments described above were all carried out with (NH₄)₂SO₄ (0.10 %) as N-source and, in agreement with earlier experiences, it was found that though the concentration of the C-com-

pound had a marked influence on resistance, it was not possible to obtain really high resistances in media with ammonia as the sole N-source.

As shown in Tables II and III substitution of ammonia by an organic N-source *can* give media in which high resistances are obtained; but, it has eventually been found that even with an organic N-source resistance depends largely on the concentration of the main C-compound. Table IV shows a series of experiments with different N-sources (viz. ammonia, glycin, glutamic acid and the muscle extract previously described) and it is seen that not only in the case of ammonia does the concentration of citrate influence the results to a very great extent. With all 4 N-compounds we find that high concentrations of citrate has qualitatively the same lowering effect on resistance: *within the same experiment the highest citrate concentration always gives the culture with lowest resistance.*

Table IV, however, also shows that the percentages arrived at in

Table IV

Resistance Determinations on Bacteria grown in Media with Varying Citrate Concentrations and Different N-Sources.

N-sources	Citrate-conc.	Survival Percentages in Experiments Nos.:			
		I	II	III	IV
$(\text{NH}_4)_2\text{SO}_4$ 0.10 %	2.00 %	1	1	1	2, 2, 5
	0.63 %	19	—	—	—
	0.20 %	19	24	13	—
	0.05 %	—	—	—	55, 21, 40
Glycin 0.12 %	2.00 %	3	—	—	—
	0.63 %	81	—	—	—
	0.20 %	67	—	—	—
Glutamic acid, HCL 0.40 %	2.00 %	2	53*)	3	—
	0.63 %	37	—	—	—
	0.20 %	75	ca. 100*)	71	—
Same + $(\text{NH}_4)_2\text{SO}_4$ 0.10 %	2.00 %	—	6	7	—
	0.20 %	—	30	14	—
Muscle extract (cf. Table II), 10 % per vol.	2.00 %	16	—	—	—
	0.63 %	67	—	—	—
	0.20 %	81	—	—	—

In this experiment 3 more (decreasing) concentrations of glutamic acid were used on both citrate-levels. In the group with 2 % Citrate the percentages 53, 59, 57 & 64 were found, while the group with 0.2 % citrate gave the percentages ca. 100, ca. 100, 92 and 87 respectively. Thus, *within* experiment II the usual rise in resistance is found on lowering the citrate concentration; but, for unknown reasons, this experiment differs considerably from nos. I & III with respect to percentage level.

media of identical composition may vary somewhat from one experiment to the other; for this reason it is necessary to stress that direct comparisons between survival percentages can only be made safely within the same experiment, i. e. when all inoculations have been made from the same culture.

Control experiments have shown that neither lag phase nor growth rate change to any great extent within the range of citrate and ammonia concentrations investigated; not until muscle extract, casein hydrolysate or a similar complex factor is added to lag and growth rate shift from the figures characteristic of the synthetic media (about 2 and 1 hours respectively) to the figures found when growth takes place in one of the usual broth media (about 1 to 1.5 and 0.4 hours respectively). — Other control experiments have proved that the low resistances obtained in the media with high concentrations of citrate do not result from increased demands for O_2 or CO_2 under the given growth conditions. Neither is the drop in resistance caused by changes in the pH of the extracellular fluid (the amount of buffer in these media has been found to be sufficient to keep the pH constant throughout the growth period).

We have now seen that loss of resistance in synthetic media does only take place under certain conditions; *i. e. to obtain loss of and not to preserve resistance special growth conditions must be exacted.* — In consequence of this observation further experiments were made with a medium of the following composition: normal Na-citrate 2.0 %, $(NH_4)_2SO_4$ 0.1 % and $MgSO_4$ 0.01 % in n/15 phosphate buffer with pH = 7.0, (owing to the high concentration of citrate the actual pH of this medium is about 7.3).

In the first place the relation between resistance and age of culture was examined: inoculating with highly resistant bacteria from a fresh bouillon culture »survival percentages« above 100 were regularly found early in the logarithmic growth phase. As mentioned above, such findings indicate that active growth, which in the case of a young culture may take place during 30 minutes treatment with serum, overshadows the bactericidal effect. — For technical reasons such an effect has to be reckoned with when the concentration of citrate in the culture is as high as 2 %. In the early growth phase when the bacterial density is about 10^6 /ml. resistance was determined by mixing *undiluted* culture with serum (equal volumes) which gives a citrate concentration of 1 % in the complement system. This concentration is known to inhibit complement activity strongly (3). With decreasing concentrations of citrate inhibition weakens and is hardly perceptible with concentrations below 0.1 %. Thus, when the bacterial density is above ca. 10^6 /ml. the culture can be diluted to such an extent that the citrate transferred to the test system no longer influences the bactericidal process. After 7–8 hours when this density is reached the measured resistances, which are no longer influenced by citrate, drop rapidly and within

2—4 hours they attain low and constant values with about 5 % surviving bacteria. — Essentially similar results are obtained when bacteria with acquired low resistance are used for inoculation: the same initial phase with percentages above 100 is registered, the drop in resistance occurs at the same time and the same low values are attained, (see Table V, Fig. A).

Table V, Figure A.

Resistance Determinations at Different Times on Bacteria grown in Synthetic Medium with 2 % Citrate or in Culture Filtrate.

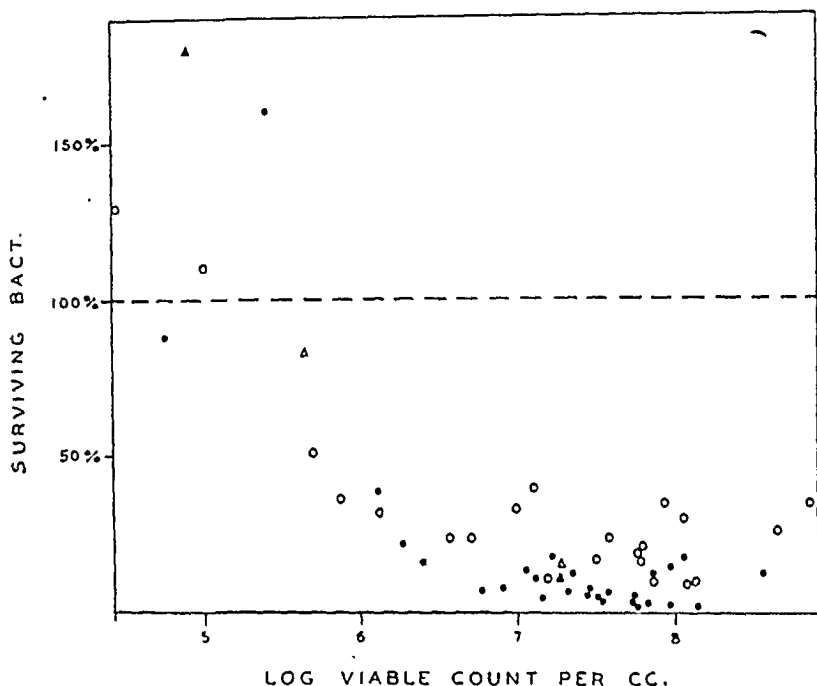


Figure A: Dots represent values from cultures in fresh citrate medium (2 %); 3 different experiments are included. — Open rings represent values from cultures in sterile filtrate from a grown out culture in the same medium. (see text); 2 different experiments included.

△ indicate values obtained after inoculation with resistant bacteria from a bouillon culture. ▲ indicate values from cultures inoculated with bacteria with acquired low resistance.

The highest citrate concentration actualized in the complement system (1 %) will as mentioned lower the bactericidal activity very considerably and may thus alone account for the high percentages obtained from the youngest cultures; there are, however, other factors which may be involved. — Firstly, it is well known that in the early phase of growth unusually large bacteria are formed. Experiments with *E. coli* have shown that these bulky cells differ from the smaller forms mainly in their size, some of the basic metabolic activities of

the two cell-forms being identical per mg. of substance (4). As it must be assumed that the bactericidal activity of the large complement molecules is a surface activity, cells of great bulk may be more resistant than the smaller forms which in proportion to their surface contain less live substance and nutrients. Secondly, it has been observed that the contents of nucleotides are increased in the cells in the early

Table V, Figure B.

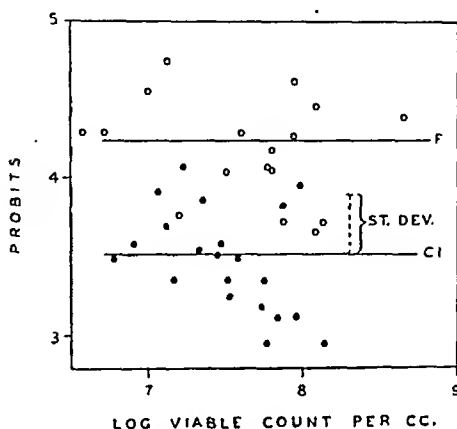


Figure B: Signatures as in Fig. A. Percentages transformed to Probits. — The horizontal lines marked F and Ci indicate the mean Probit values in the filtrate and the citrate groups respectively. — The two groups show normal distributions and have a common standard deviation ($= 0.38$ Probit units) which is marked out in the Figure.

growth phase (5). Whether this difference between cells in young and in older cultures or the above suggested »scale effect« are of any importance for the resistance to complement can not be decided at present and we shall not go into further discussion of these problems. The important conclusion to be drawn from the experiments represented in Table V is that with inocula of about 10^3 /ml. a low and constant level of resistance is reached after 12–14 hours when the density of the cultures is $\geq 10^6$ /ml. Thus the loss of resistance in media with high concentrations of citrate occurs relatively early and is not dependant upon the higher densities later obtained in such cultures.

In order to see if, at the time when this density has been reached in the cultures, any substances of importance for the development of resistance has been accumulated the following experiment was set up: A 48 hours culture in 2 % citrate-medium with a density of 1.6×10^8 /ml. and the usual low resistance was filtered and the sterile filtrate, which with respect to pH, citrate and ammonia contents, was practically as before inoculation, was used as growth medium alongside with fresh citrate medium in a new experiment. This experiment (Table V) shows two series of resistance determinations at different times during growth in the filtrate and in fresh citrate-medium respectively.

It is seen immediately that loss of resistance occurs at the same time in both series and that, apart from some fluctuations, the level of resistance reached in the filtrate is a little *higher* than in the fresh citrate-substrate. This means that no filtrable substance that may accumulate in the medium during growth can be responsible for the development of low resistance:

The first examination of Table V (Fig. A) shows that the survival percentages obtained from the cultures in fresh citrate-medium differ less among themselves than do the percentages from the filtrate-cultures. This difference is, however, a result of the technique employed: As shown earlier by the writer (3), *the bacteria in a grown out culture use to be practically normally distributed with respect to resistance to complement*; this means that to a given change in resistance does *not* correspond a constant difference in survival percentage — a change from 50 % to 40 % is *c. g.* of much less importance than a change from 15—5 %. This of course makes quantitative estimations of results as expressed in percentages uncertain, but the difficulty is readily overcome if Probits are substituted for percentages. After this transformation (Table V, Fig. B) a given difference on the ordinate express a constant change in resistance irrespective of the level; and, as Fig. B shows, the Probits corresponding to the filtrate and the citrate-percentages respectively are distributed around their mean values in the same way. On further analysis it is actually found that the two groups of Probits show normal distributions and have *the same standard deviation* (= 0.38 Probit units).

Discussion:

Trying to describe what happens when the bacteria during growth lose resistance it is useful to consider the different mechanisms that may be involved in the process: —

As to the effect of ammonia the most likely explanation seems to be that the free ammonia in the media in a general way inhibits transamination processes (6), and thus indirectly slows down the synthesis of substances containing amino acids that can only be synthesized themselves through transamination. Using media with varying concentrations of ammonia it has, however, not been possible to demonstrate significant differences between the resistances obtained with high and low ammonia concentrations respectively. The reason for this failure may be that ammonia inhibits in very low concentrations, or that the ammonium concentration *inside* the cells is relatively independent of that of the external fluid. There is at present no evidence favouring either of these possibilities.

Concerning the very pronounced effect of changes in the concentration of the C-compound several different mechanisms have to be discussed:

(1) An adaptation may take place to the carbon source employed; the result being that enzyme-systems that readily use the given substrate are developed at the expense of others; such a change in enzymic composition might cause the observed fall in resistance.

(2 a & b) A gradual accumulation may take place of metabolites which normally are broken down and which inhibit the synthesis of the resistance determining substance. (a) Such metabolites may diffuse rapidly through the bacterial cell membrane, meaning that the degree of inhibition will be determined by the concentration of inhibitor in the total milieu; (b) the inhibiting metabolites may diffuse slowly or not at all in which case it is the concentration in the *internal* environment of the cells which determines the effect.

(3) Finally it is possible that in the given substrate the bacteria are unable to keep up a sufficiently rapid synthesis of the substance on which resistance depends. This may be the case irrespective of changes in proportions of existing enzyme-systems, development of new ones or accumulation of inhibiting substances.

Some of these possible mechanisms can be tested experimentally: —

ad (1) A change in the enzymatic composition of the cells is likely to take place rather slowly, and in any case the behavior of bacteria adapted to the synthetic medium should be different from that of non-adapted bacteria. It is, however, found that if two samples of citrate-medium are inoculated (a) from 48 hours culture in citrate medium and (b) from a fresh bouillon culture, both subcultures lose resistance in exactly the same way, (see Table V, Fig. A). This observation together with the rapidness with which a citrate culture regains full resistance on transfer to bouillon (Table I) indicate that adaptive changes *are not a necessary step* on the way to formation of bacteria with low resistance. On the other hand adaptation may be responsible for the fact that in serial cultures in substrates with relatively low concentrations of C-compound a gradual loss of resistance is found, (see Table I).

ad (2 a) If loss of resistance depended on accumulation of inhibiting substances in the medium a very rapid loss were to be expected on growing bacteria in a culture filtrate in which the supposed substance had already reached a high concentration.

Table IV shows that loss of resistance in such filtrates occurs at the same time as in fresh citrate medium *and that the lowest resistances are obtained in the fresh media*. This means that extracellular metabolites can not be responsible for the loss of resistance.

ad (2 b) It is, at the present state of investigations, very difficult to distinguish between this possibility and the following more vaguely defined mechanism (3). — We have seen how a slight and very »un-specific« change in the composition of the media, i. e. the shift from low to high concentration of C-compound, is sufficient to some extent to inhibit the synthesis of the factor on which resistance depends. It seems not unlikely that a high concentration of e. g. citrate, with correspondingly intense metabolism of this substrate, gives rise to an abnormal accumulation of breakdown products *inside* the cells.

ad (3) This last mechanism is, of course, the one on which we must

fall back in case the above possibilities have been excluded. As accumulation of inhibiting metabolites inside the cells can not be excluded, we shall only point out here that an intensification of the conversion of citrate in itself may have an inhibiting effect on other processes through competition for a common co-faktor. With a mechanism of this kind neither adaption nor accumulation of inhibiting metabolites would have to be considered as preliminary to the loss of resistance.

Conclusions.

The results submitted in this paper can be summarized as follows:

Chemically defined media have been selected which favour a rapid and great fall in the resistance of the bacteria to the action of complement. This action is registered by means of the percentage of bacteria surviving treatment with normal, fresh serum, and it has been shown that such survival percentages can best be compared after transformation of the percentages to Probits.

The properties of the selected media on which the loss of resistance depends have been studied: the most important factor seems to be the concentration of the C-compound, as high concentrations of this component usually give very low resistances irrespective of the kind of N-source employed. The changes in concentration of C-compound examined have little influence on lag phase or growth rate. — With a given concentration of C-compound it has been found that a shift from organic to inorganic N-source lowers resistance.

The observed drop in resistance is caused neither by adaptation of the bacteria to the C-compound employed nor by accumulation of inhibiting substances in the extracellular fluid. From the above discussion it will be clear that so far nothing can be said about the way in which the loss of resistance occurs.

Summary:

The influence of the composition of synthetic media on the resistance of the bacteria formed to the action of complement is investigated. — The idea of a specific factor necessary for the development of resistance has to be abandoned. It is shown that, on the contrary, to obtain loss of resistance special growth conditions are needed; *viz.* a high concentration of carbon source should be chosen and ammonia should be preferred as N-source.

The different mechanisms are discussed through which the loss of resistance may occur.

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MEINICKE'S REACTION

1. MODIFIED AND USED AS A RAPID SLIDE TEST.
2. USED AS A QUANTITATIVE REACTION IN COMPARISON
WITH W. R.

By *Johns. Krittlingen*.

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During the last few years the treatment of syphilis has, as a result of new remedies and new methods, made great progress. Kendell, Rose and Simpson have used one-day courses of treatment (arsenoxide + fever) with, it is stated, good therapeutic results. Other methods using intensive arseno-therapy, and lasting from 5 - 20 days, became much more widespread during the war-years and to-day it is possible and should be quite safe with combined penicillin-arsenoxide courses to give a 1- 2 week routine treatment of early lues.

This short massive treatment has shown that the syphilitic antibodies do not disappear immediately after all the spirochaetes may be presumed to be destroyed; but this happens gradually, within a variable length of time. The general supposition is, that the longer the duration of infection before treatment the longer it takes for the organism to cease producing antihodies.

A number of authorities regard it as being of inestimable help in the follow-up of patients treated for syphilis to follow the titration of the anti-hodies with quantitative sero-reactions. In the U. S. A. and the U. K. it has therefore become the usual procedure to employ quantitative sero-reactions.

These quantitative reactions, however, mean considerably more laboratory work and for this reason it has become customary to use one or other of the exclusion tests, which relatively easily and safely separate the positive sera, thus enabling them to be given further examination.

An exclusion-test, whether a precipitation- or an agglutination-reaction, should fulfil the following requirements:

1. It should be sufficiently sensitive to react positively, if possible, with all the specific positive sera one is able to find with any of the other reactions carried out in the same laboratory.
2. It should be simple and straight-forward to carry out and easy to read.
3. It should give as few false reactions as possible.

As a brief selection of the numerous exclusion tests now used, one might mention:

1. Berger: Agglutination Test.
2. Klein: Microscopic Slide Precipitation Test for Diagnosis and Exclusion of Syphilis.
3. Kahn: Presumptive Test with Serum.
4. F. Rappaport and F. Eichhorn: Rapid Test for the Sero Diagnosis of Syphilis.

Most syphilis-reaction are rather complicated and the separate components of the reaction often very sensitive and labile. For this reason results with the same reaction often vary very considerably from one laboratory to another. In some countries the discrepancy between results from different laboratories has been so great that it was found necessary to institute competitions, both between the different laboratories and between the different reactions. These competitions often proved very necessary and useful, and they have eradicated a number of methods and reactions often long considered to be very reliable.

For all reactions, the preparation of the antigen is an important factor and the slightest inaccuracy may bring an otherwise good reaction into discredit. It would certainly be in the best interests of serology, therefore, if laboratories with insufficient and less-experienced assistants could obtain their antigens, and perhaps also their other reagents, from the larger central laboratories. In support of this suggestion, it may be pointed out that M. kl. R. II has, over a number of years, been utilized extensively, maintaining its position as one of the best precipitation reactions, and that the preparation of the antigen for this reaction has been restricted to some few laboratories, so that there has been little occasion to complicating »improvements«.

In this country, a number of works have been published which show that Standard M. kl. R. II came well up to the level of the best of the other reactions used. Other advantages of this reaction are that it is simple to perform and that the results are very easy to read. So far as is known, there is no serviceable method for the M. kl. R. II antigen used as a »Slide Test«.

F. Rappaport and F. Eichhorn state:

»Idé, Klein, Laughton and Meinicke have described rapid test on Slides for the Serodiagnosis of Syphilis, but these have several draw-

backs, e. g. not every positive serum can be detected; slide tests are not suitable for a large series of sera because of evaporation from the small amount of fluid on the slides etc.«

But, while aware of these objections, we thought that it would, nevertheless, be interesting to attempt to work out a micro-reaction with Meinicke antigen.

This work was carried out in this Institute. The results could then be compared with the routine reactions which were carried out — W. R. and Wadsworth Brown's reaction — and since practically all the sera came from the various departments of the University clinic in Oslo, it was a comparatively simple matter to obtain further particulars about the patients when necessary.

Reagents, Apparatus and Method.

Reagents.

1. Meinicke Antigen (Astra).
2. 3.5 % Na.Cl.solution.
3. Serum.

Apparatus.

1. Glass-plates, 12×9 cm. (washed).
2. Solid paraffin.
3. Ring Loop of thin metal thread, covered by thin cotton thread, for making paraffin rings, (diameter about 15 mm.)
4. Pasteur pipettes and a glass-rod.
5. Stand (shelf) for placing several glass slides.
6. Jar with lid large enough to contain above-mentioned stand.
7. Incubator.

Method.

Preparatory work. The glass slides are cleaned and freed from fat. Melt the paraffin and make rings with the wire loop. On the above-mentioned slide, there should be sufficient room for 20 rings. The serum samples are then brought in tube racks containing 3 rows, each of which, holding 10. As with ordinary M.K.L.R., active serum is used. Place a Pasteur pipette in each tube. Make the antigen - saline mixture in the usual way and use only salt solution without soda (main series or series 1).

One then begins on the first test by blowing a little drop of serum out of the Pasteur pipette down into the first ring on the top left-hand side of a marked slide. Blow out of the pipette (back on to the test or out into its own container) and place it in water immediately. Do the same with all the tests, and within 1—2 minutes, there will be a drop from each of the 30 tests, each in its own ring. Then, from a Pasteur pipette of suitably coarse calibre, place 2 drops of the prepared antigen - salt solution mixture into each ring. With a glass-rod, mix the serum and the antigen. If the slide is free from grease, the mixture will spread out evenly. Should there be a trace of grease left, the mixture will form a contracted drop. Wipe the glass rod (between each test) with blotting-paper, a clean towel or a sponge. When the glass-slides are finished, place them in the stand, which is then put down in a jar in the incubator. See that there is always a little water in the jar, for otherwise the test will evaporate somewhat.

The test should be incubated for 20–30 minutes and will then be ready for reading.

Reading. In strongly positive reactions, the agglutination is so clear as to be readable with the naked eye, and comes after 4—5 minutes' incubation. Otherwise, go over the tests with the microscope using dimmed illumination and a magnification of approximately 40—50. After some practice, one does not need more than a moment on each test to be able to read even the weakest positive reaction. All positive sera should be taken out and tested with an ordinary M.kl.R.II and WR, if desirable with quantitative reactions.

A slide-test with Meinicke antigen was, by this method, carried out on 3,100 sera, parallel with Wadsworth-Brown and Wassermann reactions. All the positive sera were tested with ordinary M. kl. R. II. The work was done in three series (I, II and III on respectively 1,100, 1,000 and 1,000) and it is worthwhile to show the results separately because they demonstrate clearly how much importance must be attached to experience.

As already stated, practically all the sera in Series I, II and III were taken from persons undergoing treatment for one disease or another in various departments of the University clinic, including some from known cases of syphilis.

In Series I, 229 sera in all reacted positively with one or more reactions. Of the positives, 120 (Group A) came from known syphilitics and 109 (Group B) from patients with a diagnosis other than syphilis. Of Group B, 41 (Group BI) were verified as specific reactions and 68 (Group B II) as non-specific or false. (By false reactions is meant in this case, those which have occurred as a result of the transfer of anti-bodies from a syphilitic serum to a negative serum through a technical error). The figures, corresponding to Series I, for Series II and III, will be found in Table No. I. A general survey of the results of the investigation is given in Tables no. 2, 3 and 4.

Before going on to discuss the tables, it should be pointed out that the total figure of positives includes a large number of very weak and uncertain reactions, as the tests were read without any knowledge of the clinical diagnosis. Most of the unspecific ones were such weak reactions which usually were eliminated by a re-testing of the same serum or by a new test. Wadsworth-Brown and Wassermann reactions were carried out and read by one person; Micro M. kl. R. and M. kl. R. by another (present writer) without consultation, before the results were written down.

Table No. 2 shows combinations of positive reactions — both specific and non-specific. As the purpose of the work was to examine Micro-M. Kl. R. as an exclusion-test, one will immediately observe that some sera from known cases of lues gave positive reaction with WR or Wadsw., but failed on the micro-tests. The whole question concerns two sera which only gave positive reaction with WR and five sera which only gave positive reaction with Wadsw. The five were all from treated cases of lues and gave very weak reactions. Three occur in the 1st series and two in the beginning of the 2nd series, so that it is

Table 1.

	Series	I.	II.	III.
1. Total number of tested sera	1100	1000	1000	
2. Total number of sera which gave pos. reaction with one or more antigens	229	165	219	
3. Total number of pos. sera which originated from known cases of syphilis (Group A.)	120	82	86	
4. Total number of pos. sera which originated from undiagnosed cases of syphilis and other diseases (Group B.)....	109	83	133	
5. Number of group B verified as syphilis (Group B ₁)	41	33	37	
6. Number of group B verified as nonspecific (Group B ₂)..	58	60	96	

Table 2.
Combination of positive reactions.

	Series I T. N. pos. 229				Series II T. N. pos. 165				Series III T. N. pos. 219			
	Groups				Groups				Groups			
	A	B	B ₁	B ₂	A	B	B ₁	B ₂	A	B	B ₁	B ₂
Total	120	109	41	68	82	83	33	50	86	133	37	96
Pos. 1. 2. 3. 4.	38	19	19	0	28	15	15	0	42	18	18	0
Pos. 1. 3. 4. Neg. 2.	4	2	2	0	1	0	0	0	5	1	1	0
Pos. 1. 4. Neg. 2. 3.	0	0	0	0	0	0	0	0	2	0	0	0
Pos. 1. Neg. 2. 3. 4.	1	0	0	0	0	0	0	0	1	0	0	0
Neg. 1. Pos. 2. 3. 4.	48	12	12	0	28	9	9	0	30	15	13	2
Neg. 1. 2. Pos. 3. 4.	20	3	2	1	19	10	6	4	3	6	4	2
Neg. 1. 2. 3. Pos. 4.	6	49	4	45	4	26	3	23	3	6	1	5
Neg. 1. 3. Pos. 2. 4.	0	5	1	4	0	0	0	0	0	0	0	0
Neg. 1. 3. 4. Pos. 2.	3	19	1	18	2	23	0	23	0	81	0	81

1. stands for WR.

2. » » Wadsworth B. R.

3. » » M. Kl. R. II.

4. » » Micro-M. Kl. R. (Rapid slide test.)

reasonable to suppose that on account of technical errors and uncertainty in reading, some of these have been lost. On the other hand, one cannot ignore the possibility that it may be a case of non-specific

reactions, which a large percentage of the weak positive Wadsw. reactions proved to be.

Of the two sera from known cases of syphilis which gave only positive WR, one occurs in Series I and one in Series III. In the first series, the test was taken from a treated syphilitic and gave very slight inhibition of hemolysis. Again, one must suppose that here too the discrepancy is due to a chance error.

With the test in the 3rd series, the conditions are quite different, for it was taken from an untreated case of lues. In this case, WR was very strongly positive and when this serum was mixed with negative

Table 3.

Group A (see table I) in each of the three series.
Number of positive results with each different reaction.

Group A.

	Series I T. N. pos. 120 (100 %)	Series II T. N. pos. 82 (100 %)	Series III T. N. pos. 86 (100 %)
WR.	43 (35,8 %)	29 (35,4 %)	50 (58,1 %)
Wadsw. B. R.	89 (74,2 %)	58 (70,7 %)	73 (83,7 %)
M. Kl. R. II.	110 (91,7 %)	76 (92,7 %)	80 (93 %)
Micro- M. Kl. R.	116 (96,7 %)	80 (97,6 %)	85 (98,8 %)

Table 4.

Group B (see table I) with sub-groups B₁ and B₂.
Number of positive results with each different reaction.

Group B.

	Series I		Series II		Series III	
	B ₁ T. N. 41 (100 %)	B ₂ T. N. 68 (100 %)	B ₁ T. N. 33 (100 %)	B ₂ T. N. 50 (100 %)	B ₁ T. N. 37 (100 %)	B ₂ T. N. 96 (100 %)
WR.	21 (51,2 %)	0 (0 %)	15 (45,5 %)	0 (0 %)	20 (54,5 %)	2 (2,1 %)
Wadsw. B. R.	33 (80,5 %)	22 (33,3 %)	24 (72,7 %)	23 (43 %)	33 (86,5 %)	88 (91,7 %)
M. Kl. R. II.	35 (85,4 %)	1 (1,5 %)	30 (90,9 %)	4* (8 %)	36 (97,3 %)	2 (2,7 %)
Micro- M. Kl. R.	40 (97,6 %)	50 (73,6 %)	33 (100 %)	27 (54 %)	37 (100 %)	13 (13,5 %)

*) From 4 cases of organic heart diseases.

serum, a positive micro-test resulted. This was therefore a case of prozone and immediate evidence that the micro-test was not absolutely reliable as an exclusion test — which, admittedly, no other precipitation reaction is either.

At short intervals, 3 tests were taken from this patient. On one occasion, the micro-test was quite negative, on another, weakly positive, and on a third, somewhat more strongly positive. The reason for the variable result is that the drops of serum may vary somewhat in size and the drops of antigen likewise. With the combination — 1 small drop of serum + 2 large drops of antigen — the strong serum will give a positive reaction, whilst a large drop of serum will give prozone. (This was done repeatedly as an experiment)..

Each of the 3 samples of this serum gave quite negative reaction with ordinary test-tube M. kl. R. and became positive when diluted with negative serum. Wadsw. R. acted in the same way. (In diagram I, one of the samples is diluted with negative serum, Micro-M. kl. R., test-tube M. kl. R., and WR carried out on the dilutions). Wadsw. reaction failed on several strong lues sera on account of prozone — one of the causes of the great handicap of this reaction.

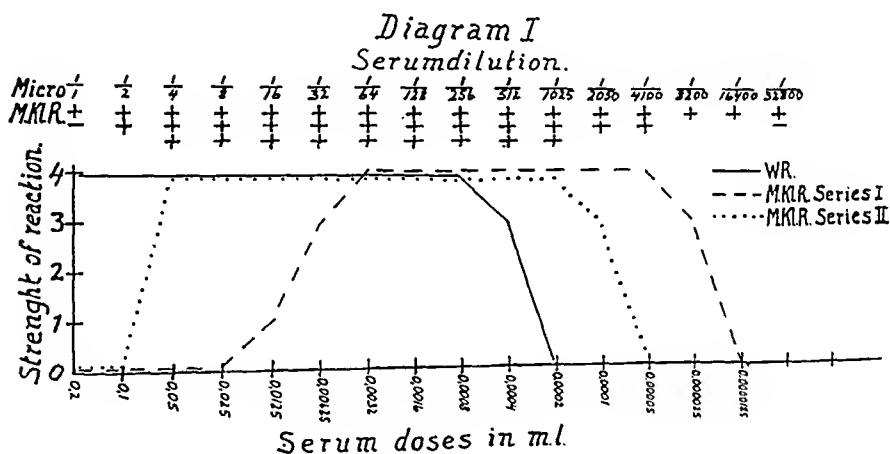
Table no. 3 shows the total number of sera from known cases of syphilis which reacted positively in one or more reactions and the number and percentage detected with the separate reactions. Micro-M. kl. R. comes first with 96.7 %—97.6 % and 98.8 % in the 1st, 2nd and 3rd Series respectively, with test tube M. kl. R. approximately 5—6 % below. Wadsw. comes in the 1st and 2nd Series approximately 20 % below test tube M. kl. R. II. In the 3rd Series, a more sensitive Wadsw. antigen was used, and the result came 10 % nearer test tube M. kl. R., but, as will be seen from *Table 3*, this antigen gave so many non-specific reactions, which were often moderately strong and sometimes strong, that it had to be considered wholly unserviceable.

The great rise in the percentage of positive WR in the 3rd Series is due to a more sensitive antigen. *Table no. 4* gives a survey of the positive sera taken from patients with different diseases, including some with undiagnosed syphilis. For each series is given the total number of sera which reacted positively and how many there were of these which were verified as specific reactions and as false or non-specific. Micro-M. kl. R. detects in the 1st Series, 97.6 % of all those verified as lues sera; in the 2nd and 3rd Series, 100 %. Ordinary M. kl. R. in selective ability is second to the micro reaction and gives the smallest number of false or non-specific reactions. Wadsw. B. R. gives, both in the 1st and 2nd series, a large number of the non-specific (false) reactions and is responsible for the majority in the 3rd series, when the more sensitive antigen was used. One observes that in the 1st series, Micro-M. kl. R. gives 73.6 % of the non-specific, while in the 3rd series, the figure has dropped to 13.5 %.

There were several reasons for the large number of false Micro-

M. kl. R. in the 1st series. A glass pencil was used in the beginning, instead of paraffin rings, to divide off the glass slide into squares, but this did not stick well, and quite often the tests flowed together somewhat. Another factor was that thin sealed glass capillaries were used instead of a thick glass rod for mixing the tests, and it was not practicable to clean these properly between the tests. The most important source of error was, however, that small drops of serum also spirted onto the adjacent rings if one blew without due care when the test was about to be placed into its ring. After a little practice, this seldom occurs, but should one find that there is a weak agglutination in several places around a strongly positive test, this may be the cause. If a drop of serum is squeezed out of the Pasteur pipette with the fingertip, this source of error is avoided.

As a final precaution against technical errors, one may set up a new test from each of the selected positive sera. In this case, one must

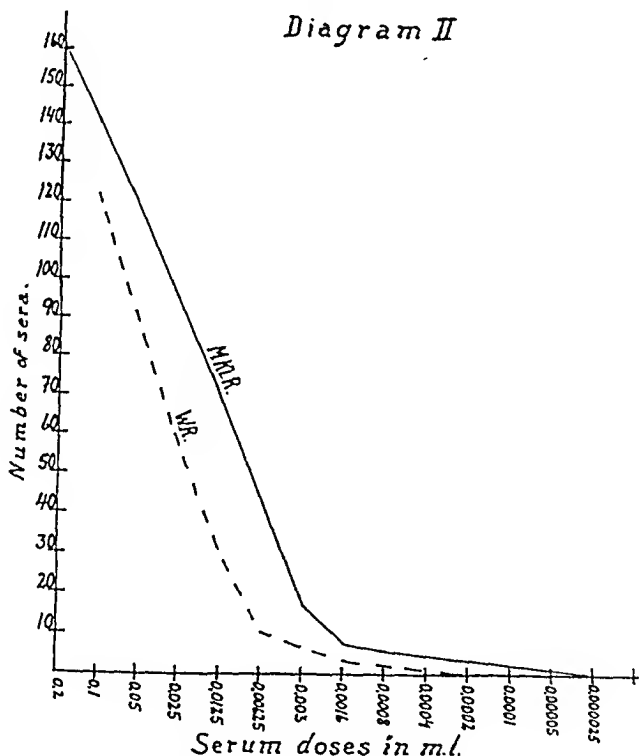


The significance of quantitative syphilis reactions has been mentioned above. During this last war, the author treated a large number of men of the Royal Norwegian Navy and Norwegian Merchant Navy with a 5-day intensive Mapharside treatment. As part of the follow-up of the patient, a number of trials were made with M. kl. R. as a quantitative reaction. The method was quite simple; the positive serum was diluted in negative serum and then prepared as ordinary M. kl. R.

The following figures were used in grading the results:

for: M. kl. R.	for: WR.
0 — No clarification;	corresponds to 100 % Lysis.
1 — Appreciable clarification;	» » 75 % »
2 — Strong »	» » 50 % »
3 — Almost complete »	» » 25 % »
4 — Complete »	» » No »

The figures for the clarification of each separate tube in the row of titrations are added up and the total is used as an expression of the contents of anti-bodies in the positive serum. For »over-strong« sera with prozone, 4 was reckoned as the value for each tube in the prozone range — (Diagram I: WR = 39, Series I M. kl. R. II = 55, Series II M. kl. R. II = 47).



Quantitative WR and M.K.I.R.II. carried out on 162 sera (see p. 777).

This method produced results which one believed to be useful. To begin with, two rows of titrations were set up and used:

Series I: Antigen mixed with 3.5 % Na. Cl. Solution, and

Series II: » » » 3.5 % Na. Cl. » + 0.01 % soda.

Series I was found to be adequate and Series II was therefore discontinued in routine tests.

In order to ascertain the relationship between quantitative M. kl. R. and quantitative WR, the following experiment was carried out in this Institute:

162 sera from known cases of syphilis, varying from weak to strongly positive were titrated in negative serum. From each tube in the row of titrations, 0.2 was used for M. kl. R. and 0.1 for WR. The result of this experiment is given in Diagram II. As will be seen, the curves showing the two reactions run fairly parallel, and one may assume that the quantitative difference between the reactivities of the M. kl. R. antigen and the WR antigen is constant from serum to serum, provided that an accurate method and the same reagents are used.

Each new antigen which is taken into use should be tried out on a number of sera parallel with the antigen it is intended to replace. If one takes into consideration the relation between the reactivities of the titrated antigens, it will be possible to obtain greater continuity in progress of successive quantitative reactions for a definite serum.

Conclusion:

1. A method for the use of Meinicke antigen in a rapid micro-reaction is described.
2. The method has been compared with WR and Wadsworth Brown reaction on 3100 sera. In one positive serum, it failed on account of prozone inhibition — a fact which shows that it was not 100 % safe as an exclusion test.
3. M. kl. R. was used as a quantitative reaction in comparison with WR. The proportion between the titres of quantitative M. kl. R. and quantitative WR seems to be fairly constant from serum to serum, provided that the same reagents and an accurate method are employed.

Since medio 1946 the rapid slide test with Meinicke antigen has been performed as routine method in this institute. All sera which did not show a definitely negative micro test, were examined with W. R. and ordinary M. kl. R. II. In all excluded sera (definitely negative micro

test) a simplified W. R. (one dose only) was done, a precaution taken to eliminate loss of positive reactions through prozone phenomenon.

Total no. of sera examined with the micro test	10730
» » » » which showed negative result	9215
<hr/>	
» » » » picked for further examination	1515
All picked sera were tested with quantitative W. R. and M. kl. R. II. Both or one of the reactions were pos. in	1263
<hr/>	
Surplus	252
<hr/>	

The clinical diagnosis of patients whose serum showed a trace or weakly positive micro test only:

Total no. of sera:	252	(100 %)
Syphilis	68	27 »
Cases under obs. in a V.D. dept.	31	13,5 »
Skin diseases	31	12,2 »
No information	22	8,7 »
Organic heart disease	10	4 »
Arthrosis or arthritis	10	4 »
Venereal diseases other than syphilis	6	2,2 »
Cephalalgia	4	1,6 »
Iridocyclitis and choroiditis	4	1,6 »
Tumores?	3	1,2 »
Under 60 other diagnoses	60	24 »

In the majority of the 252 sera the micro test was very weakly positive or showed just a trace. And usually these doubtful reactions happened to be found next to a strong positive reaction. In a smaller number the micro test was weak but definitely positive.

During the year the micro test was carried out by four different persons, (3 months each) and each of them is, before they had experience, responsible for some traces and weak reactions which must be regarded as unspecific.

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TRICHINOSIS IN GREENLAND

By *Niels B. Thorborg, Svend Tulinius, and Hans Roth.**

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Although the little round worm, *Trichinella spiralis*, and the disease due to it, called trichinosis, were not discovered before the nineteenth century, there is no doubt that infection with this parasite occurred in ancient times. In fact, it was perhaps more common in those times when mankind was carnivorous to a greater degree and lived on raw or undercooked meat.

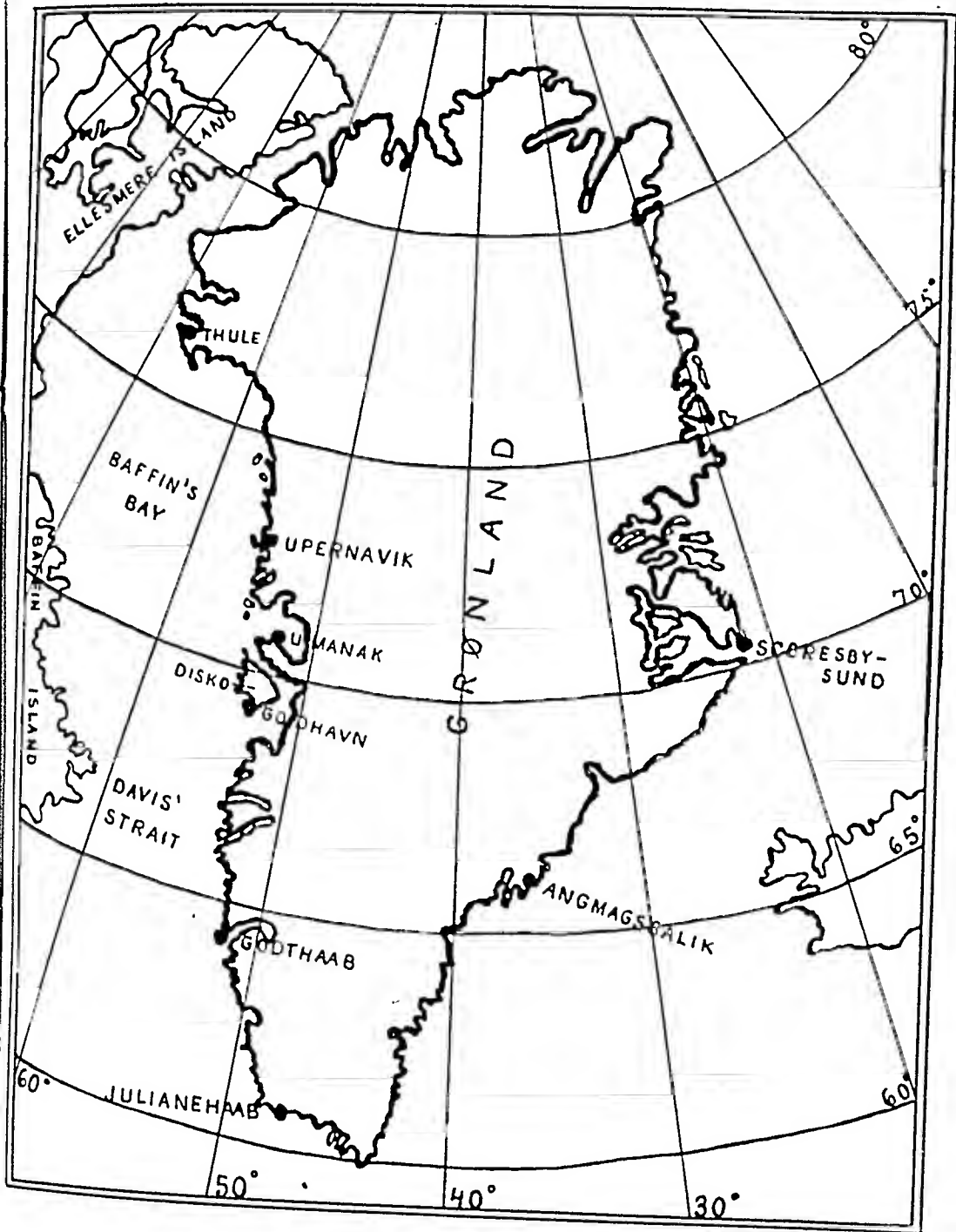
In our day trichinosis is still found with varying frequency in many countries, but as a rule the disease is considered to be limited to the temperate and subtropical zones. It seems surprising, if one considers from a purely speculative standpoint the geographical limits of the disease, that trichinosis should not be more common in arctic regions. The eating habits and diet of the native population would appear to be conducive to *Trichinella* infection; but until the time of the present investigation, no cases of infestation had been reported from the largest arctic island, Greenland.

This paper reports the details of an investigation carried out in 1947 which established that trichinosis is present in the northern part of Greenland, and that it has also occurred there in the past.

Outbreaks in 1947.

On May 11th, 1947, the State Serum Institute in Copenhagen was asked for assistance by the Greenland Government, because an epidemic of an obscure nature had occurred in the settlements around Disco Bay. 148 cases with 4 deaths had been reported from the Jakobs-havn and Kutdligssat Districts. The disease was said to be somewhat

*) The serological examinations were performed at the Department of Special Pathology and Therapy, The Royal Veterinary and Agricultural College, Copenhagen (Director: Professor H. C. Bendixen).



like paratyphoid fever. The State Serum Institute was asked to make an investigation as to the actual nature of the epidemic.

On May 17th. one of the Institute's assistants (*Thorborg*) left for Greenland, taking with him laboratory equipment for diagnosis of enteric infections.

The clinical and epidemiological observations which will be described in the following, are partly taken from the notes and oral communications of the local physicians, partly from personal observations and from information given by the native population. In many respects the data given ought to be more exhaustive, but one must consider the difficulties of medical work in Greenland. Each physician has the



responsibility for a very large district without much assistance. Means of transportation are primitive, the climate is rigorous and careful supervision of the patients is often impracticable. Furthermore, linguistic difficulties and the special mentality of the Greenlanders make the anamnestic information somewhat unreliable. The primitive and bad dwellings also make the examination of the patients difficult in many cases; even in the hospitals, the possibilities of performing laboratory examinations are poor.

From the first days of January until the middle of May, about 300 cases with 33 deaths occurred. All cases, with the exception of two, were distributed in the districts around Disco Bay as far south as Holsteinsborg.

Geographically and chronologically the cases can be grouped into six outbreaks:

In Kekertak and Ikorfat: 37 cases (3 deaths), 6th Jan.—middle of Feb. 1947.

In Egedesminde: 25—30 cases, about 1st Feb.—about 1st April 1947.

In Jakobshavn: 32 cases (3 deaths), 16th March—12th May 1947.

In Christianshaab and Claushavn: 13 cases (1 death), April 1947.

In Holsteinsborg, Avssakutak, Umanarssuk, and Sarkak plus Sukkertoppen: 50 plus 2 cases (15 deaths), 14th March—10th May 1947.

In Kntdligssat: 131 cases (11 deaths), 27th March—30th May 1947.

Picture of the disease.

Onset. In most cases the illness started with gradually increasing malaise, headache, fatigue, pains in the trunk and the limbs, and a moderate fever. In some cases diarrhoea occurred; now and then sore throat or other catarrhal symptoms were observed. In some instances the disease began with chills, high fever, headache, severe pains of the limbs as well as vomiting and diarrhoea.

Fever. In the first days the temperature gradually rose to 39—40° C, often followed by intermittent temperature (about 38—39° C) for a variable period, terminated by lysis. The patients as a rule had a slight fever for about 1—2 weeks, before the temperature returned to normal. In some patients the fever was high from the beginning, while in others there was moderate fever during the whole time they were ill. A few mild cases were afebrile.

Exanthema. About 80 % of the patients showed a rash of varying form at the beginning of the illness or some days afterwards. In some cases, however, it was not seen until later. The form most often seen was represented by non-itching, pink spots, 2—4 mm in size, which first appeared on the extremities, particularly on the legs, and then extended to the trunk. In other cases there was an intense, maculopapular, morbilliform exanthema all over the body and the face. Sometimes it took scarlatina form, and some patients had strongly itching urticaria which was sometimes recurrent.

The rash persisted from a few days to 2—3 weeks; as a rule its duration was one week.

Edema. In the first days of the second week after the onset, but often earlier, and in a few cases even starting from the onset, edema of the face or of the extremities appeared. In the most severe cases, the edema was generalised; two patients showed very great edema of the penis and scrotum. During the course of the disease the edema seemed more to become an edema of myocardial origin. In some of the mildest cases the edema was transient, persisting no longer than a few days.

Muscular symptoms. Almost all patients complained of weakness and pains in the muscles, particularly in those of the back and the ex-

tremities, but also in those of the face. In several cases the pains caused trismus and difficulty in swallowing. It was characteristic that severely affected patients were forced to lie supine with the arms somewhat flexed. Any movement was attended by pain. Several patients complained of pains behind the sternum or in the uppermost part of the epigastrium (Myocarditis? Diaphragmatic pains?).

Gastro-intestinal symptoms. Approximately half of the patients had diarrhoea at some time during the disease, either at the beginning or later on; generally it persisted for 1—2 days, but it often recurred, and in a few persons it lasted persistently for several weeks. The stools were bloody in several cases; in two patients haemorrhage of the bowel was so intense that it must be considered the direct cause of death.

Cardialgia, abdominal pains of a more vague character, constipation and distension occurred frequently.

No enlargement of the spleen or the liver was observed. One patient had transient icterus.

Cardio-vascular symptoms. The blood pressure was low (systolic pressure less than 100 mm) in those cases where it was determined. The pulse was relatively slow in some cases, but in many patients it was more rapid than might have been expected in comparison with temperature. This phenomenon must be considered as a sign of myocardial involvement which in a number of cases was also manifested by stasis of the lungs, hypostatic edema and cardiac dilation.

Among the deaths were several cases of sudden death, which no doubt were due to myocarditis.

Other cardio-vascular symptoms included cutaneous, intestinal, and vaginal haemorrhage. Gangrene of one foot, presumably as a consequence of arterial thrombosis, was seen in 1 patient.

Pulmonary symptoms. A number of patients had a cough, but no other pulmonary symptoms.

Many of the severely affected patients showed dyspnoea due to myocardial insufficiency, which was often complicated by pneumonia.

Neurologic symptoms. Headache and insomnia were among the most frequent complaints.

The severely affected patients showed apathy.

A 12-year-old girl, on the last day before her death, was restless and confused, with incessant vomiting, which symptoms were possibly of cerebral origin.

A few patients complained of disturbed vision. Ptosis and paresis of the abducens nerve was observed in one patient.

General course of disease. The disease varied in severity from very mild to fatally-terminating cases; the period of illness varied from a few days to 12 weeks. In children it generally had a milder course than in adults, but, on the other hand, death also occurred among children.

In the mildest cases the patients did not show other symptoms than a transient rash or a slight edema of the face, possibly slight fever.

In serious cases, the general condition of the patient was poor with severe muscle pains, generalised edema and myocarditis.

The clinical picture was different in different localities. Thus for example only mild or moderate cases occurred in Egedesminde, while severe cases were especially seen in Avssakutak. In conformity with this, the mortality rate varied from 0 to 37 %. The average mortality rate for the epidemic as a whole was about 10 % (Table 1).

Death occurred in most cases after about 4 weeks' illness; in some cases, however, even earlier, and in 2 cases as late as 3 months after the onset. The immediate cause of death usually appeared to be myocarditis, often complicated with pneumonia; in 2 cases, however, the cause of death was intestinal haemorrhage.

The convalescent period was rather long, accompanied by fatigue, pains in the muscles, and edema of the feet and ankles. Many patients were obviously anaemic during convalescence.

On the whole, the symptoms corresponded generally with the usual description of the disease, although certain irregularities were noticed.

The conventional classification of the disease into 3 stages, viz. 1st stage — with predominant gastro-intestinal symptoms, 2nd stage — with fever, edema of the face and general intoxication, 3rd stage — with predominant muscular symptoms was observed only in some of the cases. In reality the 1st stage was often lacking altogether, and no definite boundary could be seen between the 2nd and 3rd stages.

The cutaneous symptoms with exanthema seem to have been much more prominent in our cases than is generally described. Frequently the patients called on the doctor because of rash, without any other essential symptoms. Edema of the eyelids which is described as particularly characteristic, seldom occurred as an isolated phenomenon. Edema of face was rather more localised to the region over the cheekbones or over the masseters; on the whole, edema of the face alone was not more frequent than edema of the hands or the lower extremities.

Myocardial involvement apparently occurred very frequently; but as there were no possibilities of taking electro-cardiograms, this question cannot be determined accurately.

Epidemiology.

Only the native population was afflicted. There was no difference in the distribution between the two sexes. The youngest patient was 2 years, the eldest 63. Contagion from person to person was never determined. In most places the disease was thought to have some connection with the hunting of walrus and with the consumption of walrus-meat. A more detailed discussion of the possible sources of infection will be given later.

Diagnosis.

It is not difficult, with the symptoms described above to diagnose »Trichinosis«, provided the general picture of the disease has been kept in mind. In reality however the situation proved to be much more complicated.

At the time of the investigation of the epidemic, no one had a complete picture of the course of the disease in the localities where it was most characteristic. The original theory that the disease was typhoid or paratyphoid was soon found to be unlikely, though laboratory examinations made in Greenland gave some support to the diagnosis of typhoid fever. The Widal test was found to be positive in some cases, and typhoid organisms were cultivated from the faeces of a single patient. This patient however was no doubt a chronic carrier.

Particularly the edema and the long interval before death suggested a parasitic disease. On the basis of information received regarding the clinical course of the outbreak in Holsteinsborg, and of the observations of the non-contagious character of the disease, the diagnosis of trichinosis could be made (*Tulinus*). This diagnosis was confirmed by the examination of a blood sample, taken on 2nd July 1947 in Denmark, from a Greenland woman who in March had had a severe attack of the disease. The serum gave a strong positive reaction by the microscopical precipitin test with living trichina larvae (*Roth*).

The diagnosis of trichinosis was later (2 to 6 months after the onset of the illness) verified by 1) differential counting of 39 blood smears, 33 of which showed eosinophilia, i. e. more than 4 % eosinophiles; 2) 24 intradermal tests with trichina antigen, 22 of which gave positive reaction; 3) serological examination of 62 blood samples brought from Greenland to Denmark, 60 of which reacted positively; and finally by 4) histologic examination of muscles from a patient who had died after 3 months' illness. The musculature was crowded with numerous trichina larvae, almost all encapsulated (Prosektor Dr. med. *Harald Gormsen*).

The serological examinations will be treated in more detail in a later paper by Alice Reyn and Hans Roth.

Diagnosis of single outbreaks.

Kekertak and Ikorfat: Out of 12 blood smears from patients who had recovered, 10 showed eosinophilia, and out of 9 serum samples, 8 gave positive reaction.

Egedesminde: All cases were mild or moderately severe. No blood samples were available from these patients, but the clinical picture was so characteristic that there can be no doubt about the correctness of the diagnosis.

Jakobshavn, Claushavn and Christianshaab: The disease was quite



Fig. 1.

Section of m. biceps. Greenland woman, having succumbed to trichinosis after 3 months' illness. Magnif. $\times 65$.

typical in all patients. Serum samples and blood smears were taken from 11 patients and showed positive seroreaction and eosinophilia in 10 cases.

Holsteinsborg and Avssakutak: Serum samples from 16 patients from Holsteinsborg and Avssakutak were all positive. Of 24 intradermal tests 22 were positive, and of 6 blood smears 5 showed eosinophilia.

Umanarssuk and Sarkak (both outposts to Holsteinsborg): 4 and 1 typical cases respectively.

Kutdligssat: Serum samples from 27 patients were all positive. 9 out of 10 blood smears showed eosinophilia. In one patient, as already mentioned, the diagnosis was verified by postmortem examination of the musculature (Fig. 1).

Sukkertoppen: In the Sukkertoppen District 2 cases occurred in April. As will be discussed later these cases were connected with the outbreak in Holsteinsborg. One of these patients died suddenly during an excursion to the mountains, after his convalescence was apparently complete. The cause of death was doubtless cardiac failure. A survey

of all known cases is given in Table 1, while Table 2 gives the dates of the occurrence of the cases in the different places. It will be seen from Table 2 that more than half of all the cases began in April. The distribution of age as determined for 204 patients is shown in Table 3.

Table 1.
Survey of the cases and the mortality rate.

	Number		Percentage of the population	Deaths	Mortality rate
	Inhab.	Cases			
Kutdligssat	930	131	14	11	8
Kekertak + Ikorfat ..	150	37	25	3	8
Jakobshavn	700	32	5	3	9
Claushavn	280	1		0	0
Christianshaab	200	12	6	1	8
Egedesminde	700	30	4	0	0
Holsteinsborg	800	15	2	4	27
Avssakutak	150	30	20	11	37
Umanarssuk + Sarkak	110	5	4	0	0
Sukkertoppen	900	2		0(1)	
about	4920	295	6	33(34)	11

Table 2.
Distribution of the cases according to locality and time.

	Jan.	Feb.	March	April	May	Total
Kutdligssat	0	0	0	112	19	131
Kekertak + Ikorfat	25	12	0	0	0	37
Jakobshavn	0	0	8	16	8	32
Claushavn	0	0	0	1	0	1
Christianshaab	0	0	0	12	0	12
Egedesminde	0	20	10	0	0	30
Holsteinsborg	0	0	5	10	0	15
Avssakutak	0	0	0	15	15	30
Umanarssuk	0	0	0	0	4	4
Sarkak	0	0	0	0	1	1
Sukkertoppen	0	0	0	2	0	2
	25	32	23	168	47	295

Table 3.
Age distribution of 204 patients.

Age	2—9	10—19	20—29	30—39	40—49	> 50 years
Number	27	60	50	31	24	12

Probable sources of infection.

The native population of North Greenland obtains its most important foods of animal origin by hunting and fishing. There are fish, birds, and sea mammals: seal, walrus, white whale, and other whales, as well as terrestrial mammals such as polar bear, reindeer, arctic fox, and Greenland hare. Besides, quite a considerable quantity of dog-meat is consumed. Jerked mutton, pork, sausage, and canned meat can be obtained from the trading posts.

The native population, however, consumes very little pork, as it is rather expensive. In the last two years before the outbreak of the infection, the pork sold in Greenland was exclusively of Danish origin and might therefore be considered as practically free from trichina infestation. An extensive system of microscopic trichina control is a rule in Denmark, and no trichinous swine have been found since 1930.

During the war, American pork and canned meat was imported to Greenland, but taking into consideration the special mentality of the Greenlanders, it seems improbable that they would have kept meat for years. As a matter of fact, all the Greenlanders who had been ill denied that they had eaten pork.

The mutton sold comes from South Greenland; it is unlikely that it would contain trichinae, since it is difficult to imagine how these herbivorous animals would acquire trichinosis. The same holds good for reindeer which, moreover, were not hunted in 1947 in the districts concerned, but only near Sukkertoppen.

Fishes and birds do not need to be taken into account considering all we know about the biology of the trichinae.

Polar bears, Greenland hares, and arctic foxes are seldom found in the districts concerned, and in the abnormally mild winter of 1946—47 hunt was poor. Furthermore, none of the patients who gave trustworthy information about food consumption had eaten meat of the aforementioned animals. A polar bear shot on May 10th near Holsteinsborg did not cause any cases.

Thus only the meat of dogs and of sea mammals are left as possible sources of infection.

From the first, it would be obvious to cast suspicion upon the *dogs*. It is wellknown that they may become infected with trichinae, and the Greenland sledge dog especially eats everything it can get hold of. The meat of dogs is considered a delicacy in Greenland, but it is not eaten to any great extent, except in cases of emergency, since the meat of old dogs is not good, and the young dogs are kept for other purposes. The theory that the dog was the source of infection fits in with the geographical distribution, as the epidemic extended just as far south as sledge dogs are used. On the other hand, it is difficult to explain why such large outbreaks occurred simultaneously; nor do the reports of food consumed lend support to the theory that meat of dogs should have caused more than a limited number of cases at

most. Out of 152 patients who gave information regarding their food, 32 had eaten dog-meat less than one month before the onset of illness.

Attention must therefore be particularly directed towards sea mammals, and among them especially the *walrus* (*Odobaeus rosmarus*).

In 1947 the hunting of walrus started in January-February. Great number of these animals gather in the Spring when they cross the Davis Strait, coming from the coast of Baffin Island. The most southern region which they visit, is the Holsteinsborg District, while great numbers cross to the Disco Bay. From here the walrus migrate northward in the early Spring along the coast up to the Thule District, where they find their principal food on the large shell-grounds in the Wolstenholme Fjord. Since the walrus is a very big animal and its meat is often eaten raw or undercooked, a single walrus might if it were infected with trichinae, be the source of many cases of trichinosis, moreover these might be severe infections, as the Greenlanders consume very considerable portions of the meat.

If we compare the different districts, the dates of the hunting of walrus, and the beginning of trichinosis, an obvious coincidence is found in most cases.

In Holsteinsborg and in Avssakutak where the Greenlanders band together for the hunt, most cases occurred 1—2 weeks after the beginning of the walrus season. Moreover, all patients here had eaten walrus-meat. In Sukkertoppen, where no walrus are hunted, the only two patients with trichinosis had, nearly 2 weeks before they took ill, eaten walrus-meat sent to them from Holsteinsborg.

In Egedesminde the epidemic also began shortly after the beginning of the hunting of walrus. Although no information was available from single patients, there is no doubt that they had eaten walrus-meat.

In Christianshaab, 5 out of 10 patients volunteered the information that they had eaten walrus, while the other 5 said they had not. The single patient from Claushavn had eaten walrus during a visit to Christianshaab.

In Jakobshavn the disease began after walrus-meat had been received in the district. Nevertheless some of the patients asserted that they had not eaten it.

In Kutdligssat, where many cases occurred, all the patients had eaten walrus-meat.

In Kekertak and Ikorfat no walrus hunting had taken place, and it was impossible that the inhabitants had obtained walrus-meat from other places. What the possible source may have been will be discussed later.

From data regarding walrus-skins purchased by the Government it is possible to get an idea of the quantities of walrus-meat which might have been available. In Holsteinsborg and Avssakutak 124 and

262 walrus-skins respectively were purchased during January—May 1947.

The history of food consumed thus strongly indicated that walrus-meat might have been the cause of most of the cases of trichinosis, since an overwhelming majority of patients had eaten walrus-meat shortly before they took ill.

Moreover, no new cases arose, after the walrus caught in the Spring had been eaten. No cases of trichinosis occurred south of the districts where walrus-meat was eaten; but on the other hand, there have been no reports of trichinosis further north, where a great number of walrus are caught. Finally a number of patients maintained that they had not eaten walrus-meat.

The way in which the walrus might acquire its possible infestation with trichinae is a most interesting question. It is generally accepted that this sea mammal lives only on shells, fishes etc. However, it must be mentioned in this connection that remnants of seals have been found in the stomachs of walrus. It is possible that a voracious animal such as the walrus will eat any kind of fresh meat or carrion. During the period of migration northwards, the walrus finds but little food and during this period may thus be considered omnivorous. When they arrive at the shell grounds, remnants of shells are exclusively found in their stomachs.

As mentioned before, the outbreak in Kekertak had an anamnesis different from the others. Because of the information received from the inhabitants, none of the previously mentioned sources could be taken into consideration. The patients there had eaten no meat of swine, dog, fox or walrus. On the other hand, in December 1946 they had consumed *white whale* (*Delphinapterus leucas*) a toothed whale, which feeds principally on fish, molluscs, and crustaceans (the first cases of trichinosis began on the 5th of January 1947). Some of the patients in Jakobshavn and Christianshaab, who had not eaten walrus, had consumed white whale. It is therefore not impossible that the white whale might also be a source of infection.

In the period immediately before the onset of the disease no other kinds of whales had been caught or consumed in the localities where trichinosis occurred.

The most common sea mammals in Greenland are seals, which are caught during the whole year in all places, both in North and South Greenland. This general distribution of seals makes it very improbable that they should be the cause of specific localised outbreaks of trichinosis. To sum up, it must be stated that it is not yet decisively clear what the actual source of infection had been. However, there is much evidence to indicate that walrus, and possibly white whale, were the most essential causes. In this case, it is natural that the Greenland sledge dog too is infected to a greater or smaller extent. Unfortunately at the time of investigation, it was not possible to obtain samples of

the meat which was directly suspected of having caused the outbreaks. In a few samples of walrus-meat, collected at random, no trichinae were found. Meat samples from all the mentioned species of mammals are now being gathered for examination for infestation with trichinae larvae.

Earlier outbreaks of trichinosis in Greenland.

As above mentioned, trichinosis has never before been recorded from Greenland. The question then arises: Is trichinosis a quite new disease in this arctic region, or has it occurred before, without being correctly diagnosed? By checking the health records from Greenland, we have found at least two earlier outbreaks which no doubt were trichinosis.

In a report of the District Health Officer in Sukkertoppen a serious outbreak is described from Holsteinsborg. This began in June 1944. Altogether 63 persons were afflicted, with fever, exanthema, edema, pains in the extremities (certainly muscular pains), headache, stiffness of the neck and the back, occasional catarrhal symptoms, and often diarrhoea. 20 patients died (a mortality rate of nearly 30 %). At the time, the disease was considered to be an atypical epidemic of typhoid fever, as the Widal test was found positive in some cases. The outbreak afflicted Holsteinsborg's outposts Avssakutak, Sarfangvak, Ikerssuk, Umanarssuk, Sarkardlet and Ivtilek. It was considered that the infection was brought to the district by the walrus hunters, who a few weeks before the onset of the epidemic had been in contact with hunters from other districts.

The investigations which were carried out in Holsteinsborg because of the new epidemic of 1947, made it possible to take blood samples from 5 persons who had been ill in 1944 as well as from 1 person who had suffered from a similar disease in 1945. All these samples were serologically positive, verifying that previous outbreaks of trichinosis had occurred.

In a report from the District Health Officer in Umanak, an outbreak which occurred at the beginning of 1933 near Nugssuak is mentioned. The disease is described as »ptomaine poisoning« due to walrus-meat. The number of cases is not recorded. No deaths occurred. Two walrus had been caught on January 12th and the first cases occurred 2 weeks after the last meat had been consumed. All those who had eaten the walrus-meat fell ill, but none of the others. The symptoms were as follows: Fever up to 40° C.; violent muscle pains, especially in the neck and in the lower extremities; edema of the whole body, but particularly of the face; diarrhoea without blood; cough and dyspnoea.

Physical examination showed the liver to be a little below the right coastal margin; sensitiveness of the musculature, particularly in the calf; now and then albumin in the urine; in a few cases ny-

stagmus and diplopia. The convalescence was long, with particular complaints of tiredness in the lower extremities. The Widal test for typhoid and paratyphoid fever was negative. Considering what we now know about the occurrence of trichinosis in Greenland, there can be no doubt that this epidemic too was trichinosis. Moreover, also on this occasion walrus-meat seems to have been the source of infection.

Several times in the reports from Greenland diseases are described which might have been trichinosis. Thus it is probable that trichinosis has already existed for a longer time in the arctic regions.

This possibility has already been indicated in two former publications which do not seem to have aroused much attention. The Canadian parasitologist *I. W. Parnell* writes in 1934: »Rather more in the realm of speculation is the part the *Trichina* worm may play in those deaths of whole families which are periodically reported among the Eskimos. Those wholesale deaths are always ascribed to »ptomaine« poisoning: without, however, any real evidence«. He continues later: »It is, however, not a normal human parasite but one essentially of flesh-eating mammals, and a large variety of wild carnivores are infected. The parasite is holarctic in its distribution, and we have found it already in Arctic Foxes and Polar Bears. Whether or not seals and walrus are natural carriers is a matter still to be decided. There is no theoretical reason why they should not be«. Also the British parasitologist *R. T. Leiper* (1938) emphasises that he had frequently found trichina larvae in the muscles of polar bears and arctic foxes, which died in the London Zoological Gardens. In his opinion, these infections had already been acquired in the arctic regions before the animals were captured. And he comes to the following interesting conclusions: »The probability that trichinosis is widespread in the North Polar region has a significance in relation to Polar exploration. The replenishing of the food supplies of Arctic explorers by fresh polar bear meat carries with it a hitherto unsuspected risk. As dogs are also susceptible to trichinosis, the feeding of infected carcasses, whether of polar bear or of other dogs, might partially explain unexpected breakdown in stamina of dog teams on Polar journeys«.

The investigations presented above give good support to the hypotheses set forward by those two authors.

After our manuscript was finished we were kindly informed by the Greenland explorer *Alwin Pedersen* that in the years just before the war, some severe outbreaks of trichinosis, due to the consumption of the meat of polar bears, occurred among the population of North-East-Siberia, more exactly on the coast of the Bering Strait, and that the Russian authorities had totally forbidden any use of polar bear as human food.

Summary.

The authors describe a series of outbreaks of trichinosis which occurred during the Spring of 1947, in the district around Disco Bay and Holsteinsborg, in Western Greenland. About 300 native Greenlanders were attacked, and 33 died of the disease. It is noted that trichinosis has not before been reported from Greenland.

The clinical picture was characterised mainly by exanthema, generalised edema, fever, muscular pains, gastro-intestinal symptoms, and myocarditis. In a considerable number of cases, the diagnosis was verified by the positive reaction of the sera to microscopic precipitin tests with living trichina larvae; by demonstration of eosinophilia in the blood picture, and by positive reaction to skin tests with trichina antigen. Microscopy of muscle of a patient who had died of the disease showed massive invasion of trichina larvae.

From the history of food consumed, the authors conclude that pork may be excluded as the source of infection. The evidence points to walrus- and dog-meat, as the actual causes of infection.

A similar epidemic at Holsteinsborg in 1944, with 63 cases and 20 deaths, at that time was thought to be typhoid and was reported as such. This outbreak, however, can now also be regarded as trichinosis, since serologic examination of these former patients demonstrated antibodies against trichinae. The authors further refer to a report from 1933, concerning an outbreak of »meat poisoning« in Nugssuak on Disco Bay which, from the description of the disease, was undoubtedly trichinosis.

There is, thus, much evidence to indicate that trichinosis has been present in the population of the Arctic for many years.

Addendum to the galley proof (Oct. 22nd 1948):

During the last year an examination of meat samples from various Greenland mammals has been going on.*) A high incidence of the parasite has been proved in sledge dogs and polar bears. Out of 54 dogs, originating from the West Greenland districts in question, not less than 41 showed infection with *Trichinella spiralis*. Out of 3 polar bears from the Thule district, 2 showed infection, and of 13 polar bears from the East Greenland coast, 4 were found to be infected. As to the sea mammals trichina larvae has been found in a single specimen of *bearded seal* (*erignathus barbatus*), a seal with almost the same living habits as the walrus. This finding proves our theory that the sea mammals may become infected with *Trichinella spiralis*.

*) These examinations have been performed at the Department of Hygiene and Bacteriology, The Royal Veterinary and Agricultural College, Copenhagen, (Director, Prof. Aage Jepsen).

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NON-SPECIFIC SEROLOGIC REACTIONS IN SYPHILIS

By *Olof Sievers.*

(Received for publication June 28th, 1948).

In connection with the analysis of certain tests, one has to decide whether a reaction is specific or not — irrespective of what kind of WR technic that is used. In a previous investigation I have tried to show the importance of closely observing the anticomplementary effect of the serum. I then paid special attention to primary atypical pneumonia and malaria, which both — according to vast experience — can provoke false conclusions when judging the Wassermann reaction. By titrating the anti-complementary effect of the sera it was found that the effect was so increased that technically false Wassermann reactions were obtained. Of the cases examined there was none with positive WR which could have been looked upon as being biologically false. A positive reaction of these cases could always be explained by the enhanced anticomplementary effect. In most cases this disturbing effect of the sera is shown by using twice as much sera in the control (for details cf. Sachs and Laubenheimer). In the cases where this serum control did not show any inhibition, the anticomplementary effect of the serum was titrated. If the Wassermann reaction has been positive in the above mentioned diseases, the anticomplementary effect noted in these titrations has always been stronger than usual.

It is a well-known fact that false results may be obtained when dealing with sera not only from cases with pneumonia and malaria but also in affections of the liver. It is then, however, mostly a question of incomplete inhibitions of the hemolysis. My investigation of possible non-specific Wassermann reaction mentioned above nowadays also includes 377 sera from patients with different affections of the liver. The clinical diagnosis for three cases was syphilis, so they need not be further discussed here. Out of the remaining 374 sera, 28 showed inhibition with WR antigen. In three of the cases the cause of the reaction could not be explained. Unfortunately I have had only in-

sufficient quantities of sera for different kinds of control. Only in one case, with 50 per cent hemolysis in the Wassermann test, has it been possible to repeat the investigation with another sample of serum from the same patient, this time with negative WR. The inhibition first noted must be considered as due to a temporary deficiency either of the serologic technic or of the sampling of blood. A non-specific WR in the strict sense of the word is here out of the question. The two cases with 80 and 90 per cent hemolysis respectively in the WR must so far be looked upon as being non-specific, as it has been impossible to make any control tests. The percentage of non-specific WR of these 377 icteric sera should then be at most 0,5. Even this figure might be too high, as the two above mentioned cases could not be tested for control. Attention is called to the incomplete manifestation with 80 and 90 per cent hemolysis respectively.

The other 25 sera which reacted with WR antigen had all of them a too strong anticomplementary effect. The ordinary serum control (with 0,1 ml serum, total volume 2,5 ml) illustrated this fact in 13 cases, but in the remaining 12 cases this was made clear only by the control which contained twice as much serum. Thus, one cannot consider these cases as being WR positive, only as having the noted increase of the anticomplementary effect.

Table 4.

	a	b	c	d
Result of thymol test	Sera with anticompl. effect	Sera without anticompl. effect	Total number	a in per cent of c
0,00—0,10	8	183	191	4,19
0,11—0,15	2	31	33	6,06
0,16—0,50	7	100	107	6,54
0,51—1,00	8	35	43	18,60
Total	25	349	374	6,68

At the Central Laboratory of the Sahlgrenska Hospital (chief: professor J. Lehmann), all these icteric sera have been examined with regard to the Meulengracht reaction, serum phosphatase, serum citric acid, the thymol test, and some of them have also been examined with the sublimate test (ad modum Stolte). It is certainly of interest to know to what extent there could be a correlation between the results of the chemical and the serological tests. The three sera that gave positive WR do not show any conformity to each other as to the results of the chemical tests. But how do the sera with anticomplementary effect react? From Olhagen's investigations it is known that changes in the globulin must somehow be connected with the anti-

complementary effect. Where my cases are concerned, no connection is found between the occurrence of an increased anticomplementary effect and different values in the Meulengracht test or the amount of serum phosphatase or citric acid. The result is slightly different when it comes to the thymol test and the sublimate test. Table 1 shows the cases divided into four groups according to the results of the thymol test. At the same time one gets the distribution of the different groups of sera with anticomplementary effect.

The increase of the percentage of sera with anticomplementary effect that is noted parallel to the increase of the thymol test cannot be just an incident. A statistical calculation (χ^2) shows that a higher value of the thymol test very likely increases the chance of an increased anticomplementary effect. A corresponding statistical investigation of the results with sublimate tests and the anticomplementary effect respectively shows that a lower value for the sublimate test (below 1.20) with certainty enhances the possibility of finding an increased anticomplementary effect. From a clinical point of view it is the infectious hepatitis and the cirrhosis, which give high values in the thymol test and low values in the sublimate test, so in practice one should be prepared for false results of the complement fixation in cases with the above mentioned diseases, while icteric changes in serum, caused by other reasons (stones, tumors etc.) do not involve this risk to the same extent. In this connection I should like to point out that the discussion refers to the anticomplementary effect, so there is a risk for false results not only with WR but with all sorts of reactions with complement fixations. Besides, one must not overlook the possibility of false results in all diseases that give clearly enhanced values of the thymol test (cf. Iversen & Raaschou).

With the hitherto discussed tests I have tried to throw light upon the question of a too sensitive WR. Finally I will discuss the question of negative WR with sera, which give positive flocculation reactions. Most investigations have shown that the flocculation tests more often than WR give false results and sometimes it is very difficult to answer the clinician's question whether the flocculation tests from a certain patient are to be looked upon as being specific or not. For these cases one can use the ordinary absorption tests or for instance Kaln's verification test. When making these tests, however, I used a different technic. Considering the fact that serologic tests sometimes give a marked reaction by means of a summation of the antibody effect of two sera, which alone do not react, I have made experiments, illustrated by the following protocol:

A WR positive serum was tested in the ordinary way with WR antigen and serum in six series with increasing dilution. The dilution for the different series was made with:

a)	physiologic salt solution						
b)	»	»	»	inactive serum no. 748 (4+1)	WR	—	
				flocculation		—	
c)	»	»	»	inactive serum no. 749 (4+1)	WR	—	
				flocculation		—	
d)	»	»	»	inactive serum no. 750 (4+1)	WR	—	
				flocculation		—	
e)	»	»	»	inactive serum no. 793 (4+1)	WR	—	
				flocculation		+	
f)	»	»	»	inactive serum no. 794 (4+1)	WR	—	
				flocculation		+	

The results of the Wassermann test of the WR positive serum in the six series is shown on table 2. (The figures give the hemolysis in per cent).

Table 2.

Serum Dilution	a	b	c	d	e	f
0,2	0	70	0	0	0	0
0,1	0	90	80	50	0	0
0,05	0	100	80	100	0	0
0,025	10	100	100	100	0	0
0,0125	90	100	100	100	0	0
0,006	100	100	100	100	10	0
NaCl.	100	100	100	100	100	100

The table shows that the positive reaction distinctly diminishes, sometimes almost fails to appear, if a WR negative serum is added to the dilution liquid, this on the presumption that the anticomplementary effect of this negative WR serum does not impede the reaction. In other cases as in e) and f) in the table, the positive reaction is distinctly increased. This increase or rather additive effect I have so far tried to show with 127 sera, and the result is presented in table 3.

As shown by the table, sera from patients with non-syphilitic diseases and from patients with fully treated syphilis never give an additive effect. Sera from patients who are being anti-syphilitically treated react differently. Out of these last mentioned 34 sera, 22 caused an increase of the reaction, while the other 12 did not. I have considered an additive effect to be proved, if a WR positive serum either reacts stronger or if it has remained unchanged after having been diluted with WR negative serum. My hitherto made experiments seem to indicate that there should be an increase of the reaction only where cases with not fully treated syphilis are concerned, and this irrespective of the stage of the disease when diagnosed for the first time.

Table 3.

Additive effect with sera from different syphilitic patients.

Diagnosis	Num-ber.	WR	Kahn	Müller	Meinicke	Additive effect
Non-syphilitic diseases	66	—	—	—	—	—
	1	—	+	+	+	—
	1	—	—	+	+	—
	5	—	—	+	+	—
	2	—	—	—	+	—
Syphilis fully treated Syphilis tested during treatment	18	—	—	—	—	—
	3	—	—	—	—	—
	12	—	+	+	+	+
	2	—	+	+	+	—
	1	—	+	+	—	+
	5	—	—	+	+	+
	3	—	—	+	+	—
	2	—	—	+	—	+
	3	—	—	+	—	—
	2	—	—	—	+	+
	1	—	—	—	+	—

In other words, should a case which shows positive flocculation reactions that are inexplicable to the clinicians, give additive effects at the same time, it should be quite justified to suspect syphilis on account of the above mentioned reasons. When an additive effect fails to appear, it is by no means significant, however, nor is one entitled to draw any conclusions as to the efficacy of the treatment, guided by the present investigation. The only value of this control is that in some cases, positive flocculation tests with all probability are due to syphilis, and that they must not be neglected.

Summary.

A positive complement fixation with WR antigen in cases of primary atypical pneumonia or malaria is generally due to an increase of the anticomplementary affect of the sera. Sera from cases with different liver affections react similarly. Here, an increase of the anticomplementary effect occurs especially when sera show enhanced values in the thymol test and low values in the sublimate test. An anticomplementary effect occurs only in about 6,6 per cent of all the examined cases. No correlation between an increased anticomplementary effect and different values in the serum phosphatase, serum citric acid, and Meulengracht test was found.

Sera with positive flocculation tests and negative WR could yield a positive reaction if they were added to a known positive serum (table 2). This so called additive effect was obtained only with sera

from syphilitic patients not fully treated (table 3), while sera from fully treated syphilitic patients and sera from non-syphilitic cases do not give this reaction. The only value of this control is that in some cases, positive flocculation tests with all probability are due to syphilis, and that they must not be neglected.

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MUCUS-SECRETING AND CYSTIC EPIDERMOID CARCINOMAS OF THE MUCOUS- AND SALIVARY GLANDS

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Introduction.

Tumours of mucous and salivary glands constitute a heterogenous group difficult to survey. The recognition and classification of typical and well-defined forms would, however, facilitate the understanding of the group as a whole. This in turn would enable one to acquire a clearer knowledge of the histogenesis and the biological types, and consequently render the prognosis of individual forms of tumour possible. As a matter of fact, some well-defined typical forms have already been recognized, for example, the rare pure adenomas, and adenolymphomas.

In 1921 *Schilling* gave a very exhaustive description of a case of a peculiar, clearly epithelial tumour of the parotid gland. The structure of the tumour was characterized by squamous epithelium and cystic spaces lined with mucus secreting epithelial cells. In some areas mucus was formed centrally in the epidermoid columns. Similar tumours have since been described under different names by various writers, but they do not seem to have attracted much attention, »*épithéliomas à double métaplasie de la parotide*« (*Masson & Berger* 1924), »*eigenartiges Schleimdrüsen-carcinom*« (*Kamprath* 1927), »*carcinoma mucosum cysticum alveolare*« (*Snellmann* 1933). *Kunstmann* (1936), places this type of tumour in the same class as parotid adenomas. *Skorpil* (1940) was the first to define this form of tumour distinctly in an exhaustive description of eight cases of his own. He labelled the growth »*schleimbildendes Epitheliom*«. *Skorpil* found altogether 17 cases described in the literature. In the opinion

of the writer two cases of tumours of the jaw published by *Bossart* (1945) belong to the above-mentioned category. *Bossart* named this type »hypernephroides adamantinoma«. A few single cases have been published by *Lepp* (1939) and *De & Tribedi* (1939). Although this kind of tumour is not well-known and only infrequently recognised, it is nevertheless not so uncommon as the limited number of reports in the literature might induce one to believe. *Stewart, Foote & Becker* drew attention to this form of growth, which they call muco-epidermoid tumour. On perusal of about 700 tumours of the mucous and salivary glands they found 45 muco-epidermoid tumours and they estimate the frequency of this type of growth to be 5 per cent of the tumours of the mucous and salivary glands. *Bernier* (1946) has given preliminary descriptions of three cases of muco-epidermoid tumours. The tumour, however, seems to be so little known that a general review of its type and a description of 12 of the writer's cases are justified. These 12 cases have been collected from tumour material referred to the Department during the last seven years. This supports the view upheld by American authors, who maintain that this type of tumour cannot be so uncommon as its scanty mention in the literature might infer.

In the following a description will be given of the author's 12 cases, which, together with the cases from the literature, form the basis of a subsequent discussion on the histogenesis and the biological characteristics of this type of growth. Reports by earlier writers show that no unanimity has been reached in regard to the malignancy of these growths.

*Writer's Material.**

Case 1. Male. Clergyman. Born 1839. (Journ. Nr. Jubilee. Clin. Lund. 2425/41). In 1940 the patient noticed a lump immediately behind and below the left ear. The tumour grew slowly until it had assumed the size of a hazel-nut. On 18th March, 1941, the growth was operatively removed but the extirpation was probably not radical. On 31st March, 1941, radium tubes were inserted in the residual part of the tumour, which was then as large as a hazel nut and fluctuant. On insertion an amber-coloured fluid oozed out through the needle pricks. On 28th April, 1941, pat. was given short distance, low-voltage rtgn. treatment of the tumour, which was still cystic. Biopsy of the wall of the cyst revealed no microscopic rests. Neither did repeated follow-up examinations of the following years divulge any signs of recurrence.

* All the cases described here were treated at the King Gustafs Jubilee-clinic, Lund, where I was allowed to inspect the records with the kind permission of the chief Bertil Ebenius. In some of the old cases the only material available consisted of frozen sections. In those cases in which more material was available paraffined sections were taken and examined with the following stains: Htx-V.-Gieson, Htx-eosin, mucicarmine and Weigert's orcein stain. Furthermore, in six cases frozen sections stained with Scharlach R were examined.

But in Sept. the patient noticed a swelling of the jugular glands. Examination disclosed a general enlargement of the lymph nodes. The microscopic examination of an extirpated jugular gland showed »The whole of the lymph node consists of densely located uniform lymphocytes with round nuclei and scanty cytoplasm. The normal lymphatic tissues are suppressed. No mitoses. The picture agree to that typical of lymphadenosis«. Examination showed the blood count to be 11000 white corpuscles per cubic millimetre, the differential count being 31 p.c. lymphocytes. X-ray treatment was resumed. Clin. diag.: Lymphatic leukemia. Still in September 1947 no local recurrence in the vicinity of the left ear was observable.

A microscopic examination of the primary tumour showed: In a sclerotic connective tissue a number of strands and columns of large and polygonal epithelial cells with eosinophile cytoplasm and round nuclei with distinct nucleoles. The cells were sharply defined. No intercellular bridges were to be observed, but in appearance the cells reminded one strongly of squamous

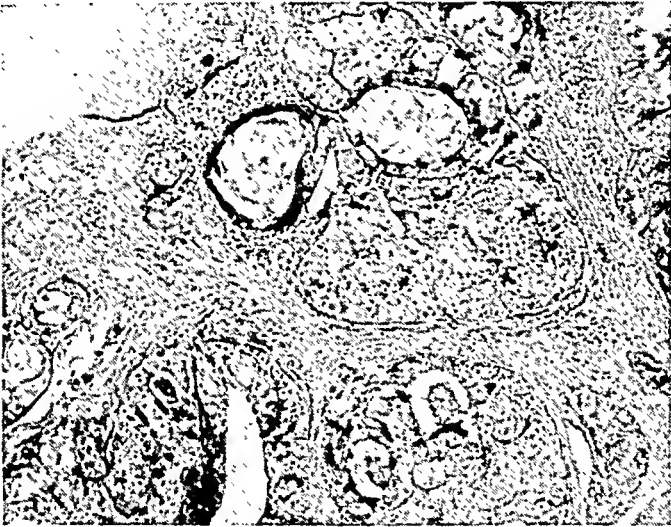


Fig. 1.

Case 1. Invasively growing solid epithelial formations with development of numerous mucus-filled cysts, some of which are lined with goblet cells. Approx. 1 : 75.

epithelium. In some cells vacuoles containing mucoid secretion were observable, and several cells were full of mucus with the nuclei forced hard against the periphery. Cysts filled with mucoid secretion and lined with typical goblet cells were to be seen inside the epithelial strands. (Fig. 1). The epithelial formations had expanded and infiltrated the stroma, which contained a large number of polymorphonuclear leukocytes. The stroma cells displayed an abundance of blood pigment.

Case 2. Man. Born 1903. Clerk. (Journ. No. Jubilee Clin. Lund. 3811/43). Since Christmas 1940 patient had noticed a gradually growing lump behind his right ear. On 12th June, 1941 the tumour was removed. The growth was cystic, and its contents were viscous. It had grown deeply inwards and the operator believed that the growth had not been completely extirpated. Pat. was given post-operative X-ray treatment and no signs of a return of the growth were visible before July 1943, when he noticed a swelling behind his

right ear. Biopsy divulged the presence of a neoplastic tissue of the same type as was previously diagnosed at the initial examination. The pat. was given telerradium treatment, but clinically no regression could be recorded. On 17th Sept. 1943 the tumour, which was located anteriorly of the mastoid process and which had infiltrated the sternocleido-mastoid muscle, was surgically removed. Radium tubes were applied in the wound cavity, which gradually healed. Pat. was followed-up and at last examination on 5.11.47, showed no signs of recurrence.



Fig. 2.

Case 2. Invading epithelial columns manifest in a sclerotic stroma with inflammatory deposit of cells. Approx. 1:75.

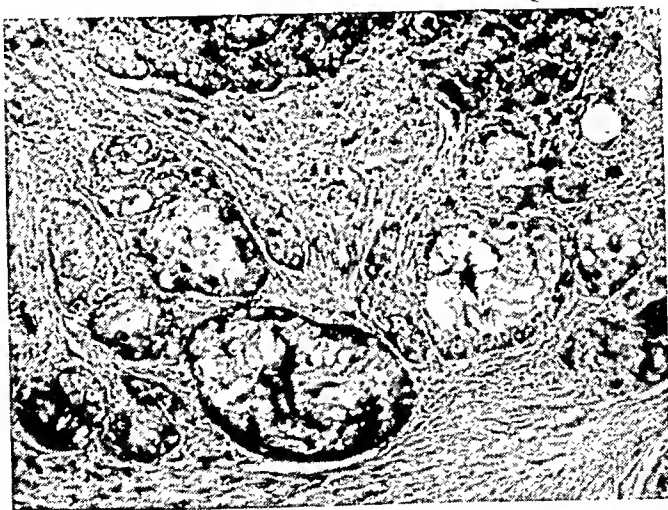


Fig. 3.

Case 2. Within this portion the growth is mainly cystic. Approx. 1:75.

*) Sections from the primary tumour were unavailable, but according to the description they exhibited the same histological picture as the relapses.

A microscopic examination was made of sections of both recurrent tumours of 1943.*) Both growths had a sclerotic, abundant stroma in which were observed richly infiltrating epithelial strands consisting of epidermal cells, and in some areas, distinct intercellular bridges were observed. The cells showed a moderate degree of polymorphism. Mitoses were not discernible. Inside the cellular columns were all transitions from cells filled more or less with mucoid secretion, to fully developed goblet cells. It was not infrequent that the latter line cysts filled with mucoid secretion. (See Figs. 2 and 3). Staining visualized numerous small fat droplets inside the neoplastic cells. In the stroma there were many polymorphonuclear leukocytes and round cells. In some areas deposits of blood pigments were also demonstrable.

Case 3. Married woman. Born 1921. (Journ. Nr. 730/47. Jubilee Clin. Lund). Since about 1943 the patient had noticed a hardened lump about the size of a pea near the angle of the jaw. In Jan. 1947 the growth was removed, but probably not radically. On 31st March 1947 a tumour 1.5 by 1.5 cm. and growing inwards behind the angle of the jaw was discovered under the scar



Fig. 4.

Case 3. Part of a large cyst is visible in right hand top corner. Invasive squamous epithelia strands, some of which are transformed into cysts, are distinguishable in the fibrous wall at bottom. Approx. 1:15.

of the first operation. After telerradium treatment an operation was performed on 30th April 1947, when the growth was electro-cauterized. The tumour had grown in towards the carotid artery. Radium tubes were applied in the operation cavity. Facial paresis occurred after the operation. At examination on 18th Sept. 1947 the wound had healed, and no signs of a recurrence were detectable.

A microscopic examination of the primary tumour showed that it had a cystic wall. The cyst was lined with stratified epidermoid epithelium containing a number of mucus-filled cells, some of which reminded one of goblet cells. The cyst had a fibrous wall exhibiting a number of infiltrating strands of epithelium, composed of epidermoid epithelial cells. Inside these strands small cystic formations filled with mucoid secretion were discernible. (See Fig. 4). Around the epithelial strands was a moderate deposit of polymorphonuclear leukocytes.

The picture of the secondary growth with its numerous strands of epidermoid epithelium in which the formation of cystic spaces, containing mucoid secretion, and lined with goblet cells were visible was substantially similar to that of the primary tumour. Mucicarmine stained the mucoid secretion very red. The growth exhibited moderate cellular polymorphism. No mitoses were observable.

Case 4. Man. Born 1900. Forester. (Journ. Nr. Jubilee Clin. Lund. 3731/45). Since the age of six the pat. had suffered from chronic left otitis, which constantly re-appeared. On 27th April, 1945, a tumour was removed from the left auditory canal. The tumour soon returned and by the time of a second examination on 17th July 1945, it had filled the whole of the auditory canal. Behind the ear was a walnut-sized growth. Clinically, the growth seemed to be malignant. On 23rd July, 1945 the (walnut-sized) tumour, which had by then diffusely infiltrated the surrounding tissues, had pierced the bottom of the acoustic duct and spread behind the mandibular joint. It was neces-



Fig. 5.

Case 4. Invasive epithelial strands most of which have developed into mucus-filled cysts, lined with goblet cells. Approx. 1:75.

sary to extirpate the facial nerve. Local radium application in the operation wound. The removal was not radical and the tumour soon recurred. On 16th Nov. 1945 a new operation was performed whereby the regio prae-mastoidea was cleared up. The tumour extended inward to the styloid process and forward to the angle of the jaw and into the parotid gland. Radium tubes were applicated in the cavity of the operation wound. The large operation wound gradually healed, and repeated follow-up examinations have hitherto divulged no signs of recurrence.

Microscopic examinations were made of the primary tumour and all recurrences. The picture was practically the same in all cases, so that the description will hold good for all. The tumour consisted of epidermoid epithelial columns whose growth infiltrated a sclerotic connective tissue which often exhibited the presence of polymorphonuclear leukocytes. Everywhere in the epithelial columns were to be seen vacuolized cells which stained red with mucicarmine. The cells were often filled with mucoid secretion and had excentrically located nuclei (See Fig. 5). In Fig. 5 we see the formation

of a number of cysts filled with mucoid secretion and lined with large goblet cells also filled with mucus. Some of the cells had disappeared and left behind them small accumulations of secretion in the tissue, but an inhibition of the tissue with this secretion was never demonstrable. The epithelial cells were rather well differentiated. The cellular nuclei were cystic with distinct nucleoles. Mitoses were rare. Staining did not divulge the presence of fat in the cells of the growth. Sometimes a direct connection was discernible between the acanthotic skin epithelium and the columns so that sprouts from the surface epithelium merged into epidermoid epithelial columns, in which cysts filled with mucoid secretion were observable.

In some areas the growth had infiltrated the inflamed tissue of the salivary glands. In conjunction with the primary operation a number of lymph nodes were extirpated. No metastases were observable in these extirpated nodes although neoplastic tissue was discernible in their immediate neighbourhood.

Case 5. Married woman, Born 1888, (Görrn, Nr. Jubilee Clin. Lund, 5722-45). In the summer of 1935 the pat. noticed a diffuse hardening under and in front of left ear. One month later she sought medical aid. Diag.: Cystic tumour. Biopsy taken. The tumour contained a clear, yellow fluid. Pat. referred to Huddal, Dept. Lund, where a tumour was found adherent to the skin and to the deeper tissues. Telerradium given, but in vain. On 4th Dec. 1935 the pat. was operated on and a sclerotic tissue with a hazel-nut sized cyst containing a haemorrhagic fluid was removed. The anterior part of the sternocleidomastoid muscle had to be removed. Radiumtubes were applied in the wound. Post-operative facial paresis occurred after the operation. Pat. given post-operative rtgn. treatment. Pat. was followed up but last exam. 27th Nov. 1937 showed no signs of recurrence.

A microscopic examination divulged an epithelioid, distinctly invasive neoplasm growing in sclerotic connective tissue and infiltrating the parotid tissues. The tumour had numerous large and small cystic spaces. The large spaces were lined with stratified squamous epithelium with scattered typical cells filled with mucoid secretion. Squamous epithelial strands were growing

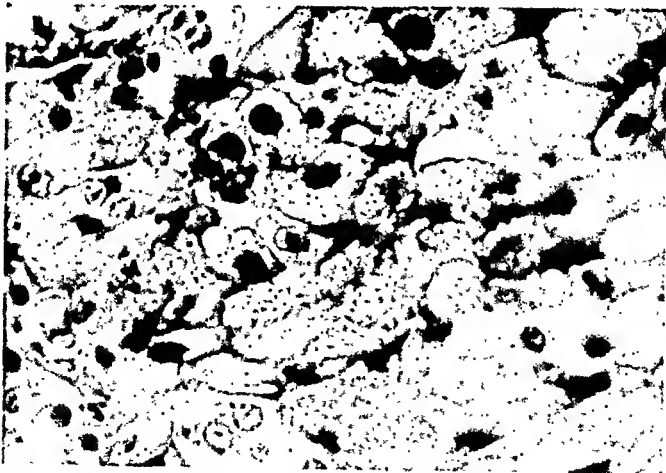


Fig. 6.

Case 5. Neoplastic cells with reticular cytoplasmic structure as in sebaceous gland cells. Approx. 1:550.

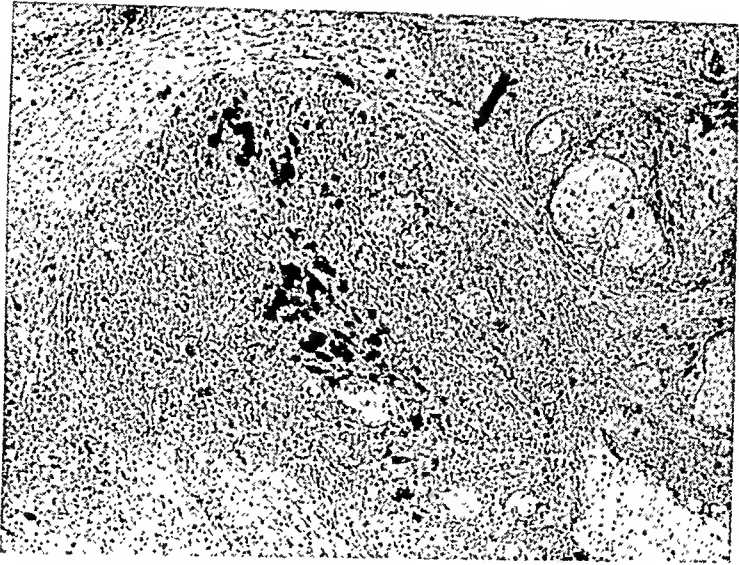


Fig. 7.

Case 5, Scharlach R stained section. An epithelial column with fat-filled cells is distinguishable in the centre. In the epithelial columns also mucus-filled cysts are visible to the right.

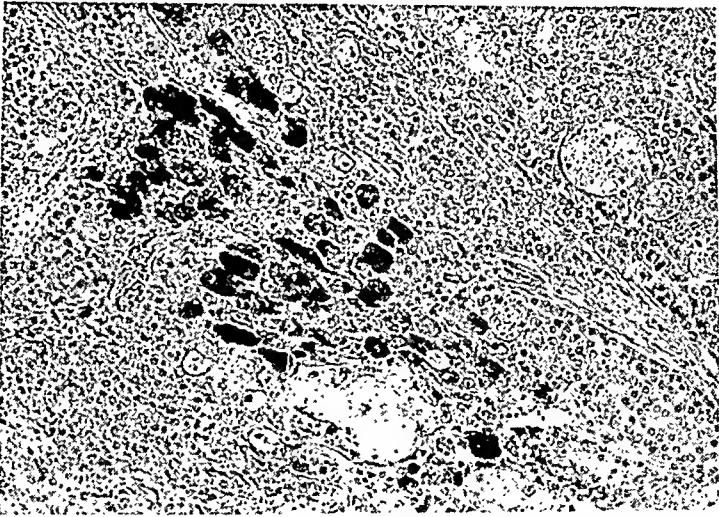


Fig. 8.

Case 5. Detail of Fig. 7.

in the sclerotic wall of the connective tissue of the large cysts. Inside these strands there were numerous cells with mucicarmine positive droplets, as well as cystic spaces filled with mucus. In some areas inside the columns in the squamous epithelium the cells had pyknotic nuclei and cytoplasm with a foamy structure, very reminiscent of the cells of sebaceous glands (see Fig. 6). Staining showed these cells to be full of numerous minute fat droplets (See Figs. 7 and 8). The stroma exhibited traces of old and fresh bleedings in some areas and an abundant deposit of leukocytes. Lymph glands, removed at operation, displayed no signs of metastases.

Case 6. Married woman. Born 1899. (Journ. Nr. Jubilee. Clinic. Lund. 1355/44). Behind left ear pat. had a swelling almost the size of a mandarine. She had noticed the tumour during the last two years and that it had grown quicker the last few months. It was hard and nodose but could be pushed against the skin and the deeper tissues. It was treated with telerradium but after 18 months it had expanded and become adherent. Operation was performed 22nd Feb. 1945. The tumour seemed to be encapsulated but ordinary intracapsular removal was impossible because the growth was too tough and could not be removed bit by bit. It had infiltrated the masseter muscle and the parotid tissue. Local radiumapplication in the wound cavity. The patient was followed up and the latest on 4th Jan. 1946., divulged no signs of a recurrence. The patient did not return for examination again until 26th Feb. 1947, which was two months after having noticed a hardening lump at the site of the primary tumour. Examination revealed the presence of a solid growth 4.5×2.5 cm. before the right acoustic duct. The tumour was very adherent. On 17th April the hard, solid tumour rich in connective tissue and extending medially inwards of the jaw, was extirpated. Pat. followed up and at last examination 18th Sept. 1947, she showed no signs of recurrence.

Microscopic examination: Recurrence showed same picture as did the primary tumour, which was a rather medullary epithelial tumour; its broad columns and strands had invaded a sclerotic connective tissue. The substance of the growth consisted substantially of moderately atypical squamous epithelial formations exhibiting no tendency to keratinize. However, some areas displayed cells filled with mucoid secretion, and some cysts lined with high goblet cells. The mucoid cells were located inside the columns of the squamous epithelium. Staining revealed no fatty deposits in the cells of the growth.

Case 7. Female. Born 1928. No occupation. (Journ. Nr. Jubilee. Clin. Lund. 66/44). Since early childhood patient had had a swelling in the parotid region. At the age of 15 pat. noticed an expansion of the growth, and examination on 3rd Sept. 1933 revealed a walnut-sized, hard fluctuant tumour connected to the parotid gland but loosely attached to the deeper tissues. On 3rd Sept. 1933 the partially cystic tumour, which contained amber-yellow fluid, was removed. Local radiumapplication in the operation wound. On 16th Nov. 1933 a recurrence was excised which was likewise cystic, and contained mucoid secretion. On 16th April 1934 a new recurrence was removed, and radium tubes were likewise applied. Pat. was followed up and when last examined on 18th Aug. 1947, she seemed to be well.

Microscopic examination. The tumour was microscopically examined on all three occasions at operation. The histological picture was the same on every occasion. In a sclerotic connective tissue adjacent the parotid gland, small squamous epithelial columns containing some cells filled with mucoid secretion were observable. Most common, however, were cysts filled with mucoid secretion and lined with goblet cells. The stroma showed a moderate cellular infiltration.

Case 8. Married woman. Born 1892. (Journ. Nr. Jubilee. Clin. Lund. 866/39). In the spring of 1937 pat. noticed a grain-sized lump immediately behind the lobe of the left ear. The growth gradually assumed the size of a pea, and it was excised on 30th March, 1938. No histological examination was performed. After about six months there appeared a recurrence, and on 1st March a hazel-nut sized tumour was removed. Pat. received post-operative telerradium treatment. On 20th July 1941 pat. was operated for cancer in the



Fig. 9.

Case 8. Cyst with numerous goblet cells in epithelial lining. Approx. 1:75.

sigmoidal colon. On 10th July 1942 a walnut-sized recurrence of the tumour was removed from the parotid region. On 8th Oct. 1942 an almond sized re-growth surrounded by hard cicatricial tissue was removed. Pat. was given telerradium treatment and no signs of recidivation were again visible until Sept. 1946 when a cystic tumour was found at the site of the previous operation. Afterwards the tumour perforated outward through the skin and an examination of a specimen biopsy taken from a fistula opening revealed a neoplastic growth. The pat. had at that time a large infiltration below the left ear and was considered inoperable, for which reason only palliative X-ray treatment was given. At examination on 16th June 1948 the tumour showed great progress with a large ulceration.

Microscopic examination. The primary tumour as well as the recurrences was microscopically examined and all the pictures were substantially the same. It showed an abundance of broad strands of squamous epithelium free of keratinization signs and located in sclerotic connective tissue. Many of these epithelial strands exhibited large cysts filled with mucoid secretion and lined with goblet cells. Also numerous larger cysts were to be seen lined

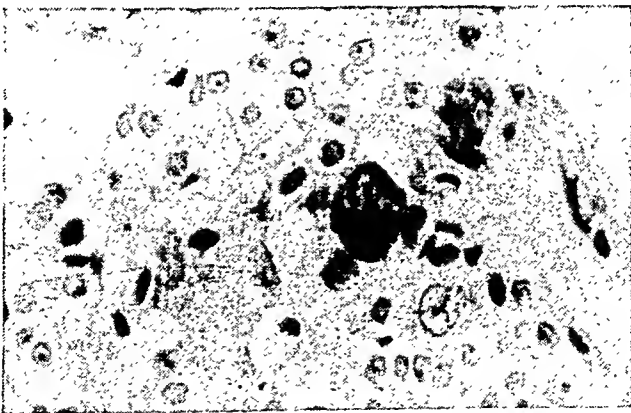


Fig. 10.

Case 8. Biopsy specimen from fistulizing tumour. Group of epidermoid-like cells among which are many cells completely filled with mucus. Approx. 1:550.

with stratified mucous epithelium with scattered and sometimes very abundant elements similar to goblet cells (See fig. 9). In its growth the tumour had distinctly infiltrated the parotid tissue, and was not clearly encapsulated. The cellular polymorphism was moderate and mitoses were rare. A small specimen biopsy scraped from a fistula of the last tumour showed accumulations of cells and scattered cells partly or completely filled with mucoid secretion (See Fig. 10).

Case 9. Female. Born 1940. (Journ. Jubilee. Clin. Lund. 3880/45). Since the beginning of 1944 the patient had noticed a tumour under the lobe of the left ear. At examination on 3rd May 1944 the tumour was walnut-sized, firm and nodose. It was loosely attached to the skin and the underlying tissues. On the 5th May 1940 the tumour, which was cystic and contained mucoid secretion, was excised. The tumour seemed to be encapsulated, but the removal was probably not quite complete. On the 11th Sept. 1944 a hazel-nut-sized, cystic neoplasm containing mucoid secretion, was removed. Radium tubes were then applied in the operation wound. In spring 1945 there was a recurrence of the tumour which fistulized through the surface of the skin. From the fistula there oozed a mucoid exudate. On the 8th June 1945 the scar and the recurrent growth were removed and radium tubes inserted. The patient has been followed-up and when last examined on 7th August, 1947 seemed to be well. The function of the facial nerve was not impaired by the operation.

A microscopic examination of the primary tumour showed numerous relatively large cysts in a pronouncedly sclerotic tissue. The cysts, which were located in the parotid tissue, were lined with stratified squamous epithelium which towards the lumen often changed into cylindrical goblet cells. The cells were uniform in shape. The epithelium often exhibited papillary formations. Cysts filled with mucoid secretion and located in sclerotic tissue dominated the picture. As a rule the cysts were lined with only a single layer of goblet cells, and squamous epithelial formations were rare (See Fig. 12).



Fig. 11.

Case 9. Recurrence in which the goblet cell formations are predominant in the papillary structures. Approx. 1 : 75.

The growth was definitely invasive. The cysts were very densely located and some of them had broken through the surface epithelium to open on the skin. The stroma was generally free of inflammatory reaction.

Case 10. Female. Born 1880. Unmarried. (Journ. Nr. Jubilee. Clin. Lund. 430/48). The patient was nursed various times during 1935—1939 for a tuberculous lymphoma on left side of the neck. The growth was operated on and the patient given X-ray treatment. In July 1947 the patient noticed a nodule under the right cheek. On 9th July 1947 a specimen biopsy of the tumour was taken and was macroscopically suggestive of cancer. The patient was

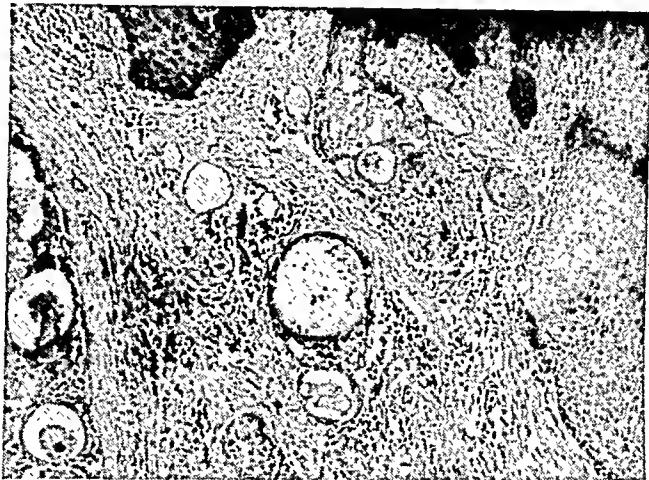


Fig. 12.

Case 11. Neoplastic formations invading up into gingival epithelial lining. Approx. 1 : 75.

then given X-ray treatment in Dec. 1947, by which time it had expanded somewhat. Patient was then referred to the Jubilee Clinic, Lund, where an examination performed on 16th Feb. 1948 revealed a tumour (2 × 4 cm.) at the site of the right sub-mandibular gland. The tumour was adherent to the skin. Metastases could not be palpated. The patient has since then received telerradium treatment, and operation is intended.

A microscopic examination of the biopsy specimen of the tumour was made. The picture showed invading strands and columns of an epithelial tumour in sclerotic connective tissue. The neoplastic cells were rather large and polygonal. Many of the cells were very light in colour and had pyknotic nuclei. In some small areas the cells resembled those of squamous epithelium, but no distinct inter-cellular bridges were discernible. The columns of the growth manifested scattered cells filled with mucoid secretion (positive reaction to mucicarmine) besides which numerous large and small cysts filled with mucoid secretion and lined with goblet-like cylindrical cells were also observable. Scattered lakes of mucoid secretion were discernible in the stroma but nowhere was the stroma diffusely imbibed with mucoid secretion. In some areas the stroma was richly invaded with round cells. Some of these accumulations of round cells displayed clearer regions of reticulum cells. Material for staining for fat was unfortunately unavailable.

Case 11. Married woman. Born 1900. (Journ. Nr. Jubilee. Clin. Lund. 3075/47). For five years the patient had noticed a tumour far back in the lower jaw. The growth began as a swelling of the gingiva. The patient had lost all her teeth in the lower jaw. During the last year the tumour had grown quicker and at examination was as large as a walnut. It had never ulcerated. On the 22nd October, 1947 the tumour was removed by Paquelin cautery. At examination on 25th November 1947 a depressed scar was found at the site of the tumour. Specimen biopsy showed microscopic, neoplastic rests.

A *microscopic examination* showed that the tumour was lined by an intact but distinctly thickened gingival epithelium, under which and sometimes ill-defined branched squamous epithelial streaks containing numerous cells filled with mucoid excretion and often with cystic spaces likewise filled with mucous excretion were manifestable (See Fig. 12). The neoplasm had infiltrated a sclerotic stroma and invaded the surface epithelium. Mucicarmine stained the secretion red. Fat staining was negative.

Case 12. Female. Born 1887. Teacher. (Journ. Jubilee Lund, 1176/45). Since 1930 the patient had a dental bridge fastened to 7—. In 1941 a lump the size of the end of a finger was noticed in the mucous membrane at this site. Radiography showed no changes in the bone. The change in the mucous membrane discovered by a clinical examination was interpreted as a typical epulis, which disappeared after irradiation and showed no signs of recurrence for a few years, during which patient was followed-up. In 1945 a flat swelling of the lower jaw was noticed on a level with 7—. Now and again the jaw was painful and purulent secretion oozed. In 1947 radiography revealed a substantial destruction of the jaw around 7—, which was extracted in the autumn of 1947. After the extraction of the tooth, the destruction of the jaw showed no signs of repair. The radiogram showed a hazel-nut-sized cystic rarefaction at the site of the extracted tooth and a substantial portion of the lingual wall of the cyst had vanished. A histological examination of a

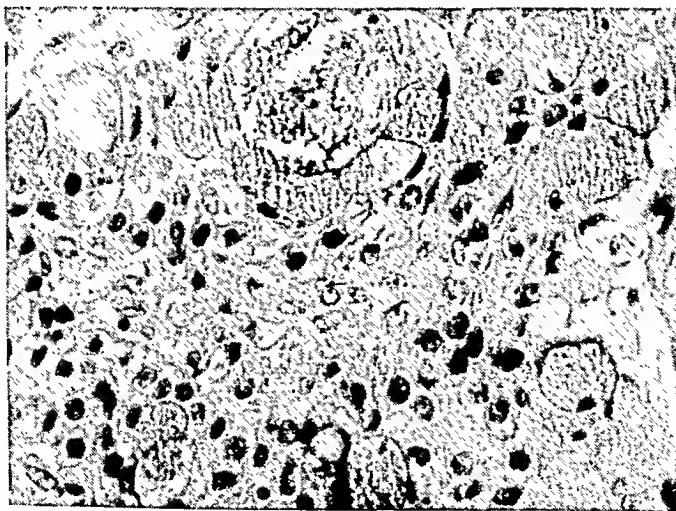


Fig. 13.

Case 12. Neoplastic group of epidermoid cells with single scattered mucus-filled cells and with cysts lined with mucous cells. Approx. 1:500.



Fig. 14.

Case 12. Isolated group of neoplastic cells. Epidermoidal character conspicuous besides which some mucus-filled cells are distinguishable. Approx. 1:500. biopsy specimen scraped out of the cavity revealed a cancerous growth. The

patient was therefore referred to the Surgical Clinic, Lund, where the lower jaw was resected on 28th January 1948. At operation a walnut-sized tumour invading the bone was discovered on the medial side of the jaw.

Microscopic examinations of the biopsy specimen taken from the destruction cavity, and of the excised tumour were performed. The histological picture was the same before and after the operation. It showed a sclerotic connective tissue containing numerous streaks or broad columns comprised of squamous epithelial, polygonal cells with rather large clear nuclei. The cytoplasm was rather clear. In some areas intercellular bridges were discernible. Mitoses were rare. Many of the cells were filled with mucoid secretion (Mucicarmine: pos.). Several various-sized cysts filled with mucoid secretion and lined with goblet-cell-like, high cell elements were manifest (See Figs. 13 and 14). Staining disclosed no fat in the cells of the tumour. The tumour was distinctly invasive. The main part of the tumour was located in the gingival tissue on the medial side of the jaw and the mucous glands immediately adjacent the tumour were apparently normal. The cavity in the jaw was filled with a rather loose connective tissue in which a few neoplastic streaks were discernible. An examination of several sub-mandibular lymph nodes showed that the latter were free from metastases.

Discussion.

In the following table a survey is given of 24*) cases referred to in the literature, and the cases of the present writer.

Occurrence. As will be apparent from the table this type of tumour may appear in any age. In one of the writer's cases the tumour

*) The 45 cases published by *Stewart, Foote* and *Becker* cannot be included here, because the publication contained insufficient data. Some obscure cases in the literature have not been included either.

was recorded in a child of 4 years (Case 26), and in another case (Case 24), a 15 year old patient, the neoplasm had existed since earliest infancy. *Skorpil* believes this type of tumour to be most common in the fifth decade of life, which may be considered probable in spite of the limited number of cases constituting the basis of his belief (see fig. 15*). The material is too small and the age distribution in the peoples from which the collection has been made is unknown so that it is not possible to draw any conclusions concerning the age incidence

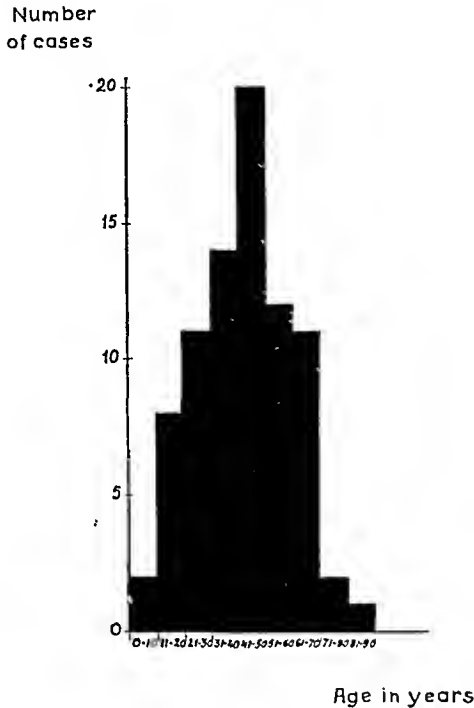


Fig. 15.

of the tumour, but nothing argues against the frequency of this type increasing with increasing age, as is the case with malignant growths.

The material is too small to permit of any more definite conclusions with regard to sex incidence**) (14 males, 20 females).

Skorpil (1940) upholds the view that this type of tumour is rare, and as only 9 cases had been published prior to 1940, his contention seemed to be correct, but later studies have, however, shown that his conclusion is debatable. The number of cases published is not a good indicator of the frequency of a tumour, because it often happens that the type of tumour has not been recognized or has been allowed to

*) This number is based on 81 cases (including the cases of Stewart et al.).

**) In the cases of Stewart et al. the tumours were »approximately equally divided between males and females«.

Nr.	Author and publication year	Location of growth	Sex and age of patient when made and sought for tumour	Length of history	Treatment	Remarks
1.	Schilling (1921)	Parotid region.	♂ 41	1 yr.	Extirpation + irradiation	No re-examination.
2.	Masson and Berger (1924)	—	? 51	3 mnths.	Irradiation	Resistant to irradiation. No re-examination.
3.	—	—	? 65	1 yr.	—	—
4.	Kamprath (1927)	Tongue	♀ 68	1½ yr.	Extirpation	Recurrence after 10 months.
5.	Snellman (1933)	Parotid region.	♂ 13	2 yrs.	Extirpation	No re-examination.
6.	Curtis and Razemon (1934)	Tongue	♀ 16	?	Extirpation	Recurrence after 5 months. Followed up 5 yrs. after re-operation but no recurrence.
7.	Kunstmann (1936)	Parotid region.	♀ 23	1 yr.	Extirpation	Recurrence after a few months. Afterwards no re-examination.
8.	—	—	♂ 35	2 yrs.	Extirpation	Recurrence after one year. Further course unknown.
9.	—	—	♀ 32	5 yrs.?	Extirpation	Symptomless 3 months after op. Case complicated by tuberculosis in lymphglands in parotid region.
10.	Lepp (1939)	Lower jaw	♀ 66	Some mnths.	Extirpation + partial resection of cheek	Free of recurrence 10 months later.

11.	De & Tribedi (1939)	Parotid region.	♂	29	3 yrs.	Extirpation	No re-examination.
12.	Skorpil	—	♂	64	4¾ yrs.	Extirpation	Exitus from other lesion after two yrs. During this time no signs of recurrence.
13.	—	Lower lip	♂	43	12 yrs.	Extirpation	No recurrence after five years.
14.	—	Sub- mandibular region.	♂	53	Some mnts.	Extirpation	Symptomless after 1 yr.
15.	—	Parotid region.	♀	43	10 yrs.	Extirpation	No recurrence after 3 months.
16.	—	—	♀	49	2 yrs.	Extirpation	Recurrence after 1 month. After re- extirpation died 3 yrs. later of cachexia.
17.	—	—	♂	20	2 yrs.	Irradiation	When questioned 14 months later pat answered that the tumour had gradually grown again.
18.	—	Tongue	♀	85	?	—	Incidental finding at autopsy. Pat. died of incarcerated hernia.
19.	—	—	♀	37	?	Extirpation	Recurrence after 7 years.
20.	Bossart (1945)	Jaw	♀	78	?	Extirpation + irradiation	At re-examination 6 months after op. no signs of recurrence.
21.	—	—	♀	49	10 weeks	Mandibular resection + irradiation	At re-examination 7 months after op. no signs of recurrence.
22.	Bernier (1946)	Parotid region.	♂	37	?	Extirpation	No re-examination.

Nr.	Author and publication year	Location of growth	Sex and age of patient when made aid sought for tumour	Length of history	Treatment	Remarks
23.	(1946) Bernier	Parotid region.	♂ 24	2 weeks	Extirpation	No re-examination.
24.	—	—	♂ 22	10 yrs.?	Extirpation	Recurrence after 1 yr. On this occasion metastase in lymphnodes.
25. (Case 1)	Linell (1948)	—	♂ 48	1 yr.	Extirpation + irradiation	Recurrence after short time. Free of recurrence 6 yrs. after irradiation. Pat. now has lymphatic leukemia.
26. (Case 2)	—	—	♂ 38	1½ yr.	Extirpation probably not radical + irradiation	Recurrence after 2 yrs. After re-extirpation + irradiation free of recurrence (time > 4 yrs.).
27. (Case 3)	—	—	♀ 26	4 yrs.	Extirpation probably not radical. New op. + irradiation	Free of recurrence (Time 6 months).
28. (Case 4)	—	—	♂ 45	?	Extirpation + irradiation in several stages	First operations probably not radical and followed by recurrences. After last op. free of recurrences. (Time 2 yrs.).
29. (Case 5)	—	—	♀ 57	1 mnth.	Irradiation + extirpation	Resistant to irradiation. After op. free of recurrence. (Time 2 yrs.).

30. (Case 6)	—	—	46	2 yrs.	Irradiation + extirpation	Resistant to irradiation. After extirpation symptomless for 2 yrs. Then recurrence. (Time 3 yrs.).
31. (Case 7)	—	—	15	15 yrs.	Extirpation + irradiation	After 2½ months recurrence which was extirpated. After 5 months new recurrence which was operated on and irradiated. Since then free of recurrence. (Time 3 yrs.).
32. (Case 8)	—	—	47	1 yr.	Extirpation + irradiation	Recurrence after 1½ yrs. After extirpation + irradiation, a new recurrence appeared after about 2½ yrs., 3 months later op. of new recurrence which was also irradiated. 4 yrs. later inoperable recurrence. Also operated for cancer of the sigmoidal colon.
33. (Case 9)	—	—	4	4-5 mnths.	Extirpation + irradiation	After 4 months recurrence which was operated on and irradiated. Since then free of recurrence. (Time 2 yrs. 2 months).
34. (Case 10)	—	Sub-mandibular region.	67	c:a 1 mnth.	Irradiation Operation intended	
35. (Case 11)	—	Gingiva (lower jaw)	47	5 yrs.	Extirpation + irradiation	Recently operated on.
36. (Case 12)	—	Gingiva (lower jaw)	60	Doubtful 3 mnths.	Extirpation	Recently operated on.

pass as one belonging to some other group such as a mixed tumour of the parotid gland, a tumour of the sweat glands, adenocarcinoma sebaceum, atypical adamantinoma etc. Examples of such erroneous diagnoses are illustrated in the material collected by the writer. *Stewart, Footes & Becker's* investigation as well as the present material show that this type of tumour is rather common.

In *Henke-Lubarsch's* manual *Lang* (1929) allocates *Schilling's* case to be benign epithelial tumours classed under the heading »adenocystoma«. It is also quite conceivable that rather many more cases of this type of tumour are described in the literature but to be found amongst other types of growth. Histological descriptions and pictures are often wanting, and it is possible and even probable that many cases of this type of growth have been observed and published in extensive collections of parotid tumours. A wider knowledge and a closer observation of the type of growth under discussion will probably show that it is relatively common and at the same time elucidate the properties and behaviour of this tumour.

This type of neoplasm is characteristic and easy to recognize, above all by its *histological structure*, which is characterized by the fact that the tumour, as pointed out by various authors (*Schilling, Masson & Berger, Skorpil, Stewart et al.*) on earlier occasions, is composed of squamous epithelial formations growing in strands and columns in a generally very sclerotic connective tissue stroma varying in quantity. Medullary forms of the growth are sometimes met with, but most of the writer's cases were rather scirrhus. Both forms i. e. medullary and scirrhus, may be exhibited by one and the same growth. As a rule squamous epithelial formations invade the connective tissue. An invasive growth of the tumour is often seen in adjacent tissue of a salivary gland or up in the skin and skin epithelium (see fig. 12). In spite of the distinctly invasive nature of the growth, many of the writer's cases had at earlier examinations been diagnosed as benign tumours. (The question of the degree of malignancy will be dealt with further down.) This is probably to be ascribed to the fact that the epithelial cells often show a high differentiation. The cells are as a rule polygonal, rather uniform in size and have medium-sized rounded nuclei with distinct nucleoles. More or less pronounced cellular polymorphism is rare and mitoses are as a rule unusual. There is no reason to doubt that the cells are epidermoid. The cellular structure is exactly the same as that of squamous epithelium and here and there intercellular bridges are distinctly visible. Earlier writers also seem to be in full agreement as to the epidermoid character of the epithelium. Cases which also show keratinization (*Stewart et al.*) are rarely met with. On the other hand the formation of mucus in the epithelial cells inside the epithelial columns is a typical and regular feature of this type of tumour. The mucus, which mucin-

earmine stains both distinctly and bright red, is visible in the form of small droplets in a few individual cells in the columns of the squamous epithelium, and as mucus lumps in cells of goblet-cell type. These cells filled with mucus may appear as isolated elements inside the columns of the squamous epithelium, as well as in the form of larger accumulations, in which case they most frequently line cystic formations filled with mucus and located inside the columns of the squamous epithelium. These cystic formations appear in varying number in different tumours and in different parts of one and the same tumour. With the growth of the cysts the surrounding squamous epithelium seems to disappear owing to pressure atrophy so that sometimes only rests of the epithelium are visible and then in the form of a thin layer of flattened cells in the periphery of the cysts. Sometimes the peripheral layer has disappeared altogether leaving behind it but a layer of cylindrical goblet cells with basally positioned, often pyknotic nuclei (see fig. 11). Also these cells disappear occasionally, leaving behind clear cell-free mucus lakes in the connective tissue. As already pointed out by *Schilling* these lakes are well-defined, no diffuse mucus imbibition on the part of the tissue ever being observable. Large cysts filled with fluid and macroscopically visible are not infrequently encountered in the tumours. As a rule these cysts contain a mucoid or amber-coloured fluid. They are generally lined with a stratified squamous epithelium with scattered goblet cells in varying number (see fig. 9). Outside this cellular layer there is as a rule a sclerotic connective tissue wall in which one generally encounters infiltrating strands of squamous epithelium with large or small cysts containing mucus. (See fig. 4). In some of the writer's cases a cellular change — apparently not observed in cases published earlier — inside the squamous epithelium columns was observed. Groups of vacuolized cells with reticular cytoplasmic structure were manifestable. The vacuoles observable in preparations imbedded in paraffin could also be shown to consist of fat (see figs. 7 and 8). This change probably explains why the diagnosis »adenocarcinoma sebaceum« was established earlier in a few of the cases. This type of cells is not a necessary occurrence in this form of tumour. Staining for fat was not possible in all of the author's cases because tumour material was not always available, but in some cases where staining could be performed, the results were negative. The significance of sebaceous gland-like cells in this type of tumour will be discussed in detail in connection with its histogenesis. In the jaw tumours published by *Bossart* (1945) glycogen was demonstrated in a number of clear cells similar to those of a hypernephroma. Such »cellules claires« were also manifest in several of the present writer's cases. Mucus secreting cells as well as epidermoid formations were also found in *Bossart's* cases. In one of the cases, staining with Best's earmine was performed and an abundance of glycogen could be demonstrated.

As a rule the stroma of the tumour consists of a sclerotic connective tissue which often shows a rich deposit of polymorphonuclear leukocytes, plasma cells or lymphocytes. Bleedings in the stroma are often observable and blood pigment deposits are common. These bleedings might possibly explain the occurrence of amber-coloured fluid present in the large cysts and, according to reports, often visible at operation.

The histological picture, as described in the preceding paragraph, of this form of tumour with scattered squamous epithelial formations and mucus secreting cells is characteristic, as already pointed out by *Schilling* and confirmed further above all by *Skorpil*. *Skorpil* would, however, carry the histological classification still further and divide this form of tumour into three different types, namely: 1: the adenomatous variant with preponderantly gland-like passages with mucus secreting epithelium, 2: the solid variant with mainly solid squamous epithelial formations (however, always with signs of metamorphic formation of mucus). 3: mixed forms between the above mentioned variants. The value of such a classification seems to me questionable for several reasons. Various parts of one and the same tumour may belong both to the adenomatous and to the solid variants. Furthermore, at the initial examination a tumour may belong to one variant and later, on recurrence, to another. Under such circumstances and at any rate as long as it is not possible to demonstrate any difference in the biological character of the various histological variants, a grouping into various types is hardly warranted.

The *histogenesis* of the type of tumour in question has been discussed earlier in the literature. That the tumours originate from the mucous and salivary glands may be considered as highly probably as they are encountered mainly in these organs. The described cases of tumours developing from the lip, the tongue and the gingiva do not contradict this assumption, as mucous glands normally exist in these regions. *Bossart* assumes in his two cases of jaw tumours that the site of origin is located in the epithelium of the oral cavity. In one of his cases he could show a direct continuity between the epithelium of the oral cavity and the neoplastic tissue around a fistula and he interprets this connection as a strong support for his opinion. Such direct relationships between surface epithelium and neoplastic tissue have also been observed by the author in some of his own cases (See Cases 4, 9, and 10, Fig. 12). Such transitions between surface epithelium and neoplastic tissue can in my opinion be explained just as well by the fact that the tumour grows up and invades the surface epithelium which then ulcerates with the consequential development of a fistula. The clinical course in Case 9 in which a subcutaneous tumour grew up into the skin and then fistulated, argues for this assumption.

It seems as if this tumour is not necessarily bound to the mucous and salivary glands, *Sikl* (1932) having described two cases of skin tu-

mours in the median of the back. Judging by the pictures published, these tumours were histologically very similar to the form under discussion. *Siki* would trace the origin of these tumours to the adnexal organs of the skin, preferably to the sweat glands. In view of this supposition the question arises whether the form of tumour under discussion might not possibly be traced to the adnexal organs of the skin, and, as most cases are met with in the parotid region, possibly to the glands of the acoustic duct. One is inclined first of all to think of that variant of sweat glands known under the name of ceruminal glands. It is, however, doubtful whether the tumours originate from these organs, as they appear in association with mucous and salivary glands distant from the acoustic duct, (tumours of the oral cavity) and because they do not resemble those tumours of the ceruminal glands reported in the literature. The cases published by *Siki* cause one to wonder whether these tumours originate from the mucous and salivary glands in the same way as those twenty or thirty tumours proceeding from various regions of the skin and which agree histologically entirely with the so-called mixed tumours originating from the mucous and salivary glands. *Schilling* believes the form of growth in question to proceed from the larger excretory ducts of the salivary glands. This opinion is also shared by several other writers. *Skorpil* believes the starting point most likely to be sought in the isthmus cells, because the latter can be differentiated in mucus secreting cells. Goblet cells are, however, existent in the larger excretory ducts, too. As far as the histogenesis is concerned, the presence of these mucus secreting cells do not teach us much because it is probably not unusual for goblet cells to develop in epidermoid epithelium. The parotid gland with its ducts develop embryologically from the oral epithelium, where there are surely developmental potentialities which possibly render

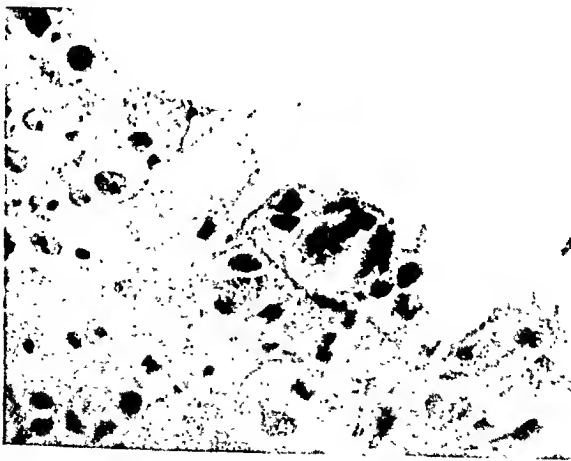


Fig. 16.

Section from wall of dental cyst. Epidermoid lining with a group of mucus filled cells. Approx. 1 : 500.

the formation of mucus secreting cells possible. As an example of this, reference is made to Fig. 16 showing a section taken from the wall of a dental cyst containing numerous cells filled with mucus and scattered in the squamous epithelial lining of the cyst. The squamous epithelium of these cysts is believed to originate from the so-called Malassez' cell-debris, remnants of the enamel organ, which is formed by a downward growth of the epithelium of the oral cavity. A perusal of preparations of 70 simple dental cysts, revealed in 4 cases a more or less abundant formation of goblet cells among the squamous epithelial cells. The formation of mucous cells is not confined solely to the squamous epithelium of the oral cavity. Fig. 17 shows a section of a der-



Fig. 17.

Section from wall of dermoid cyst from ovary. Among epidermoid cells are some mucus-filled cells. Approx. 1 : 550.

moid cyst in an ovary. In one part of the cyst is to be seen a row of cells filled with mucus which is otherwise lined entirely with keratinizing squamous epithelium. Such mucus secreting cells in the squamous epithelium were observed by the author in many cases of dermoid cysts of the ovary. Another example is the above mentioned skin tumours in the back and published by *Sikl*. Neither do the sebaceous gland-like structures in a few of the author's cases teach us much of the details of the histogenesis. Sebaceous gland structures are met with in the Stenonian duct of the parotid gland and on the interior side of the cheek (cf. the hypertrophy of these glands in Fordyce's disease). These structures may not serve as evidence for the histogenesis of the type of tumour in question proceeding from the larger excretory ducts, because the formation of typical sebaceous glands from the interlobular ducts of the parotid gland have been observed (*Hartz 1946*). Summarizing, one might be justified in saying that the histological structure of the described form of tumour may argue for its originating from the ducts of mucous and salivary glands but that the structure

does not exhibit conclusive evidence as to the particular part of the dental system in the mucous and salivary glands to which these tumours are to be traced.

From the above it will be apparent that this form of tumour has a typical histological structure, easy to recognize. A question of greater practical importance is whether this form of tumour has definite *biological characteristics* and consequential typical *clinical behaviour*.

Of the 81 cases described 51 were located in the parotid region. Seven were situated in the tongue. The material is still too limited to permit of any conclusions as to whether this form of tumour differs from others of the mucous and salivary glands as to its location of predilection. As mentioned further up, this form of tumour appears in all ages and in both sexes. The duration of the history before the patient seeks medical aid varies considerably, from one month up to 15 years. The growth of the tumour may thus be very slow and hardly noticeable for several years. According to some of the histories, however, the growth of the tumour was quicker during the last few months, and it seems as if some tumours with short histories grew rapidly from the very beginning. Clinically, the tumours resembled hard and solid neoplasms, which, at least in the beginning, could be pushed freely against the skin and the deeper tissues. Later in the course of their growth they often become adherent. On account of their tendency to develop into cysts they were not infrequently fluctuant on palpation. Sometimes they had broken through the skin and fistulated. At operation they were often ill-defined and very sclerotic indeed. Distinct capsules (as often encountered with so-called mixed tumours) were rare and a radical extirpation was often unsuccessful. In this respect the tumour differs distinctly from so-called mixed tumours, which are as a rule distinctly encapsulated, and permit intracapsular removal. The cysts were full of mucous substance or clear, amber-coloured fluid.

With regard to the biological character of the tumours it is worthy of mention that their histological picture often shows invasive growth (in all of the cases of the present material and also pointed out in many cases published earlier). In spite of the high degree of differentiation, this histological feature is very suggestive of this type of tumour being malignant. Also the pronounced tendency locally to recur argues in favour of this assumption. Of the 25 follow-ups mentioned in the table, no fewer than 15 recurred once or more often. In some of these cases the observation time was very short (some months). Some of the recurrences appeared after a short time, which was often due to an incomplete removal of the growth. However, the literature also contains reports of occurrences appearing after as long a time as seven years. Metastases of the lymph nodes are not mentioned in any of these cases, neither are remote metastases, but the material of *Stewart et al.* contains some cases with lymph node metastases and remote metastases.

Treatment was given to 34 of the cases published (one case being unexpected post mortem finding) and 31 of the cases were treated surgically and most often in combination with irradiation. Three cases treated only with irradiation did not respond to the treatment. In some of the author's cases pre-operative irradiation had no substantial effect on the size of the tumour. Neither in the rather extensive material of *Stewart et al.* did irradiation produce any notable effect in this respect. Consequently the most important therapy is presumably the extirpation of the tumour. Probably *local* application of Radium in operation wound can be of value. On account of the invasive tendency and often rather large extension of these tumours, the operations are rather disfiguring. In no fewer than 4 of the 9 cases in the parotid region in the present material was it necessary to extirpate the facial nerve, with permanent facial paresis as a result. Whether the clinical picture, course, etc. of this form of tumour differs distinctly from other tumours of the mucous and salivary glands cannot yet be decided, because the material is far too limited to be conclusive in this respect and because larger materials of tumours of the mucous and salivary glands are very heterogenetic indeed and include representatives of all sorts of forms, possibly also besides the form of tumour under discussion.

The degree of malignancy of this form of tumour is a controversial question. *Masson & Berger* believe it to be malignant. *Kunstmann* believes it to be a benign adenome, in spite of the fact that recurrences appeared in all of his cases. *Skorpil* assails this opinion and contends that it must be considered potentially malignant. *Stewart, Foote & Becker* as well as *Bernier* divide these tumours into two groups, a benign group and a malignant one. They admit, however, that there are no clear histological signs of separation and that no fixed border separating these groups can be drawn. In their group of »benign« tumours the frequency of recurrences is also high. In some of their cases a primary benign tumour seems sometimes to assume a more malignant character on recurrence. In view of the fact that the growth is as a rule invasive and as it shows such a great tendency to recur, this form of tumour ought in the opinion of the author to be designated 'malignant' in spite of the degree of differentiation, histologically often very great. In the opinion of the author it is hardly defensible to distinguish between benign and malignant types of this form of tumour. Owing to the malignancy of this tumour it seems justified to use the term 'carcinoma' and the name 'mucus secreting cystic epidermoid carcinoma' to designate its typical histological picture. This designation (*Snellman* uses the similar designation »carcinoma mucosum cysticum alveolare«) seems to me to be better than »mucus secreting epithelioma« proposed by *Skorpil*, because, as he himself points out, his suggestion may give rise to the discussion of the degree of malignancy on account of the different meanings various countries give to the term epithelioma.

Summary.

On the basis of 69 cases published earlier and 12 of his own, the author describes a special form of tumours of the mucous and salivary glands.

1. This form of tumour is histologically characterized by invasive squamous epithelial columns in which mucus secreting cells develop with the result that cysts filled with mucus and surrounded by goblet cells are often developed. Sebaceous gland-like structures often develop in the tumour.

2. Histogenetically this form of tumour can be traced to the ductal system of the mucous and salivary glands.

3. This form of tumour can appear in both sexes and in all ages, and is probably more common than previously supposed.

4. The growth has a great tendency locally to recur and both regional lymph node metastases and remote metastases have been observed. Judging by experiences hitherto, it seems as if this form of tumour does not respond to irradiation and that the most advisable treatment is radical surgical extirpation. This form of tumour ought to be considered malignant and it is suggested that it be designated *mucus secreting cystic epidermoid carcinoma*.

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CHLOROFORM-»FAT REACTIONS« WITH DIPHThERIA BACILLI

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In the course of studies of the pleuropneumonia group,¹⁾ for which after *Frosch*²⁾ I have suggested the name of micromyces, and more particularly when occupied with the relatively newly-found micromyces from female genitals (*Dienes*³⁾), I tried to clarify the pictures in direct microscopy⁴⁾ by extracting any removable fat from the colonies with the aid of various fat solvents, ether, chloroform etc. The addition of chloroform was followed by peculiar pictures: entire colonies became covered with formations like drops of fat, a phenomenon not presented by most bacteria colonies when similarly treated. When diphtheria colonies cultivated on thin — under the microscope translucent — blood plates (5 % horse blood added to broth agar) were treated, the result was a similar picture, but still more pronounced; and as the first three colony types, *gravis*, *intermedius* and *mitis*, chanced to give very strong reactions to the first two, and no reaction to the *mitis* strain, my interest in the phenomenon was awakened and an investigation on a broader basis was started.

First, something more about the phenomenon itself. On cultivating e. g. a *gravis* type on the above medium and inoculating in such a manner that the colonies will lie fairly close together, making it easy to find them by direct microscopy, we find on adding chloroform that even quite young colonies become covered with fatty drops, mostly so that they quite conceal the bacteria under them. In detail, the basic technique is the previously described direct agar microscopy;⁴⁾ a block is cut out of the medium with a knife and laid upon a slide, preferably a large one, for example 7.5×4 cm., cover glass 3.4×3.8 cm. Before adding five or six drops of chloroform, a small tuft of fat free cotton-wool spread out fine is laid over the surface and, immediately after adding four or five small drops of chloroform by means of a Pasteur

pipette, a large cover glass is placed over the preparation. Examination is made at once (powerful illumination necessary), preferably with a weak oilimmersion lens, e. g. Zeiss apochrom 60—0.85, or a powerful dry lens, moved slowly down towards the slide. After encountering a slightly misty picture one is near the colonies and one now finds them totally covered by a dense layer of drops, some of which disappear and reappear while others converge and often give the appearance of an invisible milking process which draws the drops out into long, teat-like bodies. As long as the chloroform is there, the picture remains; but as soon as it begins to disappear by evaporation from under the edge of the slide, it changes. The globules collapse like deflating balls, and at a given moment the colonies are covered by these bodies, which eventually also disappear more or less, and what remains is remarkably little conspicuous after the violent effect at the start; as the bacteria now lie, they present a picture mostly resembling the normal in direct agar microscopy.

If the surface of the medium is treated with ether before adding the chloroform, the fat picture does not appear at all. The first idea to occur was that these were drops of fat that were extracted from the colony, but fat which was only slightly soluble in chloroform. Spatially, however, the quantity is so large that so simple an explanation will not do; there could not be room for all this fat in the bacteria. I have therefore come to the conclusion that it must be fluids drawn out of the colonies and the medium in drops and covered with a fatty membrane. I have not been able to find similar observations in the chemistry of fats, but it will probably turn out to be a familiar occurrence in other connections. The fact that water plays a part in the formation of the drops seems to be evident from some small experiments. For example, on smearing a *gravis* culture from a blood-agar plate on to an agar plate and then proceeding with the same experiment as described above, we obtain a picture very similar to that of the blood-agar plate but without being so dominant, and the same is obtained by smearing the culture on to a slide — provided that it is done quickly; if we wait until the culture is dry the drop picture does not appear, but it can be reproduced by stirring the dry culture in water and quickly adding chloroform, still in the same manner with a little cotton-wool under the cover-glass to ensure the persistence of an active layer of chloroform for a sufficient length of time.

What the fats or fatty compounds are, I have not been able to determine yet; it might be mentioned that in the case of *gravis* cultures, when the cover glass is examined outside the agar block one observes at once a crystallizing of characteristic bodies resembling fatty-acid crystals. With these fatty-acid crystals, of which large quantities can be obtained by washing the surface of a slightly old culture and allowing the chloroform to evaporate, for example on a slide, it is possible to produce pictures similar to those described above, provided that

water is added to the crystals before the chloroform is added. As we shall see later when discussing the reactions of the intermedius and mitis strains, the explanation is not simply that this crystallizable substance alone conditions the formation of the drops. Another simple experiment seems to show that the combination fat — water — chloroform may produce drops which morphologically are similar to those described. If we take a test-tube which has been carefully cleansed of fat, for instance by repeated vigorous rinsings with ether, pour a little water into the bottom and add a little chloroform, and then blow through it energetically with the mouth through a Pasteur pipette, the chloroform and water will immediately separate sharply when the blowing is discontinued; but if a little fat or a small quantity of diphtheria bacilli is added before blowing, we shall quickly get a relatively durable emulsion of drops very like those seen on the colonies; very little fat is required. In most cases our test-tubes, used a few times and in our opinion thoroughly cleansed, gave a similar reaction.

Thanks to earlier virulence determinations,⁵⁾ the circumstance that the first tested mitis strain showed no fat reaction excited our imagination; for it was not impossible that such a difference in what looked like the supply of surface fat might have some influence on the behaviour of the microorganism in an infected macroorganism, similar to what happens with other functions of external microbial equipment. Let me say at once that I have been unable to demonstrate anything capable of being interpreted definitely as a connection of this sort.

It now appeared on testing a number of strains that all colonies of gravis strains and intermedius strains, eight and nine respectively, on blood agar were »fat positive« with chloroform, and I have yet to see a colony that is not.

Of 28 mitis strains, 10 were positive, but always with the difference that the number of fat drops was smaller than with the gravis and intermedius strains; another characteristic feature was that the outer margin of the colonies was nearly always bare of fat drops. With the mitis strains there was also the interesting circumstance that some, for instance our P. W. 8 and No. 5687, in culture consisted of a mixture of purely fat positive and purely fat negative. In the case of P. W. 8 it looked as if they might become the one or the other; at any rate, I did not succeed in separating them and keeping them pure, whereas this was relatively easy with No. 5687. The untreated colonies look very much the same; it is only when the chloroform is added that the difference becomes clear.

On other media, for example 10 % ascites agar, the gravis strains remain »fat positive«, whereas most intermedius and all the positive mitis strains are negative.

As I said before, with the gravis strains the fatty acid crystallization.

could always be seen outside the block of agar under the cover glass; the same applies to most intermedius strains and it is impossible to distinguish the fatty acid crystals of the two types from each other. However, there are intermedius strains which do not form these crystals. If we follow the formation intimately in all phases we shall see that with these strains there is an incipient crystallization, but that something inhibits the final process. No mitis strain forms fatty-acid crystals. When gravis strains are cultivated on ascites agar they remain »fat positive«, whereas most intermedius and all positive mitis strains remain negative. And here, in connection with what I have said above regarding a possible connection between »fat positivity« and fatty-acid crystallization, it should be pointed out that two intermedius strains which lost their fat positivity on ascites agar, nevertheless gave rise here to the formation of fatty acid crystallization. Thus any connection there may be must presumably be relatively more complicated than was indicated by the testing of the gravis strains.

I have said that it was possible to separate mitis strain 5687 into »fat positive« and »fat negative«. Both were toxin producers in vitro, and it was tempting to examine whether it was possible to demonstrate a qualitative difference in infection experiments. Employing the same technique as that described in an earlier work,⁵⁾ a number of quite young guinea-pigs, weighing about 165 g., were injected intracutaneously with 10 and 100 millions respectively in 0.1 c. c. The small doses caused but little reaction. With the large doses we obtained relatively rapidly progressing intoxications which killed the animals in the course of a few days. The reactions with the fat positive were perhaps a little stronger and a little quicker than with the fat negative, but there was no marked difference.

In order to set up infections which perhaps were more like human infections we tried another technique. The animals (still quite young ones just able to fend for themselves) were shaved on a small area on one side of the abdomen just below the thorax. The razor was wielded with a rather heavy hand so that the skin showed small red spots without bleeding, and the bacteria were rubbed vigorously into the skin with cotton-wool on a stick and dipped into a 24 hour blood-agar culture. A piece of dry gauze was laid over, and an adhesive tape 3 cm. wide all round the body kept the gauze in position during the experiment. This technique must probably have been used before, but nevertheless it was surprising to see that in this manner it was easy to produce the entire classical picture of diphtheria intoxication in the guinea-pigs, with extensive haemorrhagic subcutaneous oedema, dark red adrenals and fatal termination. It was also interesting that as a rule it was possible to cultivate diphtheria bacilli from the skin only, not from subcutis, despite the fatal termination, this being one more observation in support of the most usual experience that the ability of the diphtheria bacillus to generalize in the guinea-pigs is

extremely small; it was also curious, and pointing in the same direction, that generally the animals died just as quickly, i. e. after three or four days, when they were infected cutaneously as when they were given an intracutaneous injection of 100 million bacilli.

Here again there was no great difference between the positive and the negative variants. Perhaps the reactions caused by the fat-positive variant were slightly more pronounced; but, as stated, the difference was small. On cultivating from the infected animals I always obtained the same variant as that used in the experiments. Toxin smearing and toxin-gauze bandaging gave only superficial reaction, quite different from the reaction to smearing with diphtheria bacilli. Shaving alone caused the formation merely of a brief, quite superficial crust.

In parentheses I may add that it surprised me to find that the gravis strains, all older laboratory strains, in a dose of 10 millions intracutaneously could not kill the animals quickly, in contrast to what we found in our earlier experiments with strains newly cultivated from a severe gravis epidemic. It will be of interest to follow this up. It was also interesting to see that some strains, for instance an intermedium strain, were apparently more active intracutaneously than cutaneously as compared with several other strains, both mitis and intermedium. Naturally, it makes judgment more uncertain that it is impossible to determine quantitatively the primary cutaneous dose.

In conclusion I would merely say that outside of the diphtheria bacillus group I have found no bacteria presenting quite the same fat-picture, and most of the angular growing, coryne-form bacilli are negative. Some bacteria present a reaction with drops which appear very slowly and gradually cover the colonies; this is true e. g. of many pneumococci and α -haemolytic streptococci; tubercle bacilli also give a considerable drop reaction, but of quite a different appearance from that of colonies of diphtheria bacilli.

It was very interesting to test a number of strains resembling diphtheria bacilli which *Poulsen*⁶⁾ described and which could not be classified as diphtheria bacilli, in spite of a certain antigen fellowship. Two of these, XVI and XVII, were distinctly fat positive, and one, XVII, gave a distinct cutaneous and subcutaneous reaction with the above-described mode of infection, but was quite unable to provoke a pathological picture after the intra- and subcutaneous injection even of large doses of culture.

Summary.

Colonies of diphtheria bacilli cultivated on blood-agar often present a curious fat reaction produced with the aid of chloroform. The various types differ somewhat, gravis and intermedium strains always showing a positive reaction on certain media, whereas many mitis strains are inactive under similar conditions of growth. Some mitis strains could

be separated into positive and negative strains which retain these properties after animal passage. Tested on guinea-pigs, no definite difference could be found between positive and negative variants in a pathogenetic sense.

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